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AN IMMUNOLOGICAL AND AUTOMATED CYTOLOGICAL ANALYSIS OF CHICKENS SELECTED FOR PROGRESSION OR REGRESSION OF ROUS SARCOMA VIRUS-INDUCED TUMORS

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University of New Hampshire PH.D. 1979

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BY

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This thesis represents, as do most, several years of scientific investigation aimed toward the solution of a particular problem. And while more questions were formulated than were answered by this endeavor, it has been rewarding to work among the dedicated, unique and cheerful individuals who comprised the Microbiology Department during my stay.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

AN IMMUNOLOGICAL AND AUTOMATED CYTOLOGICAL ANALYSIS OF CHICKENS SELECTED FOR PROGRESSION OR REGRESSION OF ROUS SARCOMA VIRUS-INDUCED TUMORS

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Immunological and cytological comparisons were made of chickens genetically selected for progression or regression of Rous sarcoma virus (RSV)-induced tumors. F_2 and F_3 progeny chickens of the major histocompatibility complex (MHC) and B-blood group allotype B^2B^2 usually regressed RSV-induced tumors, while B^5B^5 hosts almost invariably died of progressive tumors. B^2B^2 chickens were immune to secondary tumor formation at 12 days following induction of a primary tumor; B^5B^5 animals were not. Thirty-three percent of B^2B^2 birds with anti-tumor immunity had detectable virus-neutralizing antibody at 12 days post inoculation (PI). No neutralizing activity was found in sera from B^5B^5 hosts.

No significant difference in number of lymphocytes in circulating blood, or in stimulation of lymphocytes by the mitogens concanavalin A (con A) and phytohemagglutinin (PHA) was observed between the two genotypes.

A method was developed to preserve the fluorescence characteristics of acridine orange (AO) stained peripheral

leukocytes for at least 5 days. Cytophotometric analysis of AO stained samples indicated that a subpopulation of peripheral leukocytes from animals with large tumors, regardless of B-haplotype, had an abnormally high intensity of red fluorescence. Since these leukocytes were non-adherent, agranulocytic and mononuclear, they are presumed to be lymphoid cells.

CHAPTER I

INTRODUCTION

That genes of the major histocompatibility complex (MHC) are important regulators of immune responses is well established (for a recent review, see Katz and Benacerraf, 1976). Early studies in mice linked genetic control of antibody production with the MHC (McDevitt and Sela, 1965), and recently, tumor development has been modified with products of MHC genes (Greene, Dorf, Pierres and Benacerraf, 1977). In the chicken, the MHC is also known as the B-locus, since it includes genes which code for the B-erythrocyte antigens. The chicken B-locus has an influence on serum complement levels (Chanh, Benedict and Abplanalp, 1976), antibody production (Pevzner, Nordskog and Kaeberle, 1975; Benedict, Pollard, Morrow, Abplanalp, Maurer and Briles, 1975), and skin graft rejection (Shierman and Nordskog, 1961).

Collins et al. (1977) have shown that chickens with the homozygous B-genotype B^2B^2 usually spontaneously regress RSV-induced tumors; B^5B^5 chickens, however, develop progressing tumors which result in early death of the animal. That an immune response (Ir) gene(s) residing in the chicken MHC might be responsible for the observed differences in tumor development was proposed.

Additional studies have examined chickens with progressing or regressing tumors for differences in: a) migration

inhibition of macrophages (Cotter, 1973); b) anti-tumor cytoxicity of lymphocytes (Archambault, 1976; McGrail, 1977); c) ultrastructure of lymphocytes and tumor cells (Archambault, 1976); d) blocking of anti-tumor lymphocyte-mediated immunity by serum factors (McGrail, 1977); e) serum protein differences (Cucchiara, 1976); and f) lymphocyte differences following acridine orange-staining (Cucchiara, 1976).

Cucchiara (1976) observed a unique serum protein and red fluorescence of acridine orange-stained lymphocytes in samples from tumor-bearing, but not from tumor-free animals. He concluded that the observed red fluorescence was due to RNA coating the lymphocyte surface since RNase treatment or repeated washing removed the red fluorescence, while DNase and protease had no effect.

The present study was undertaken to further delineate the source of the red fluorescence and the function of red fluorescing cells. Additional experiments compared anti-tumor immunity, serum proteins, circulating lymphocyte density, and stimulation of lymphocytes by mitogens in B^2B^2 vs. B^5B^5 chickens.

CHAPTER II

REVIEW OF THE LITERATURE

THE ROLE OF THE MHC IN IMMUNOLOGICAL REACTIONS

MOUSE

The major histocompatibility complex (MHC) is a group of closely linked genes which was first recognized for its control over acceptance or rejection of tissue allografts (for a review, see Klein, 1975). While an MHC has been detected in all mammalian species studied (Paul and Benacerraf, 1977), that of the mouse, called the H-2 complex, has been most thoroughly characterized. H-2 influence on antibody synthesis, mixed leukocyte reactions (MLR's), graft versus host reactions (GVHR's), anamnestic responses, delayed hypersensitivity and serum complement levels has been extensively documented (Shreffler and David, 1975). Susceptibility to viral oncogenesis was also clearly influenced by an H-2 associated gene, called Rgv-1 (resistance to gross virus-1; Lilly, 1966; Tennent and Snell, 1968).

Specific immune response (Ir) genes, which map within the MHC, are now recognized to exert positive (helper) and negative (suppressor) influence on immune responses. Separate subclasses of thymus-derived (T) lymphocytes mediate this helper or suppressor activity (Cantor, Shen and Boyse, 1976; Murphy, Herzenberg, Okumura, Herzenberg and McDevitt, 1976;

Jandinsky, Cantor, Tadakuma, Peavy and Pierce, 1976) in the mouse and probably in all mammals.

Lilly (1972) and Lilly and Pincus (1973) have postulated that Rgv-1 may be an Ir gene that influences the level of immune response to virus-induced tumor associated antigens (TAA's). Data on levels of antibody produced by mice of different H-2 haplotypes to Gross and Friend tumor associated antigens support this hypothesis (Aoki, Boyse, and Old, 1966; Lilly, 1976).

CHICKEN

Mapping of the MHC in the chicken is less complete than in the mouse due to fewer available congenic strains which differ at the MHC. However, current data indicate that avian and mammalian MHC's have similar origins and functions.

The avian B (blood group) antigens are clearly analogous to the mammalian major histocompatibility antigens (MHA's) in their apparent molecular weight (about 40,000) and association with a smaller polypeptide (B_2-microglobulin) of molecular weight 11,500 (Ziegler and Pink, 1975; Ziegler and Pink, 1976). Furthermore, the amino-terminal regions of mammalian and avian MHA's contain homologous amino acid sequences (Huser, Ziegler, Knecht and Pink, 1978).

Biological properties attributed to both mouse and chicken MHC include: specification for antigens involved in graft rejection, graft versus host reactions, and leukocyte identification; response level in MLR's; serological responsiveness to defined antigens; total serum complement levels; and

susceptibility to oncogenic viruses (Shreffler and David, 1975; Hala, Vilhelmova and Hartmanova, 1977; Morrow and Abplanalp, 1977).

INFLUENCE OF THE MHC ON TUMOR REGRESSION

MOUSE

Two mechanisms have been suggested by which an H-2 linked gene might influence tumor progression or regression. First, an Ir gene, possibly identical to Rgv-1, might determine the level of response to tumor cells. In support of this, Sato, Boyse, Aoki, Iritani and Old (1973) have shown that transplanted leukemia cells grow unimpeded in Balb/c (susceptible) recipients, but are strongly rejected by F_1 hybrids of Balb/c and C57BL (resistant) mice. Only rejector mice produced cytotoxic antibodies specific to the leukemia cells.

A second hypothesis proposed by Freedman and Lilly (1975) involves the H-2 gene Rfv-1, which confers resistance to Friend virus-induced leukemogenesis. Tumors were induced *in vivo* in mice of 3 different H-2 haplotypes (Freedman, Lilly and Steeves, 1975). Tumor cells were then explanted *in vitro* and assayed for virus production, virus-envelope antigen (VEA), and the ability to induce cytotoxic antibodies in competent hosts. Two lines of cells continued to produce virus and VEA, and to stimulate production of both virus-neutralizing and cytotoxic antibody throughout 150 passages. A third line remained VEA positive but became non-producing after 20 passages and was associated with production of virus-neutralizing but

not cytotoxic antibody. Therefore, Rfv-1 may determine whether or not tumor cells continue to express the virus-associated antigen(s) against which the immune response is directed. Other viral antigen(s) (e.g. VEA) may be unaffected by Rfv-1.

CHICKEN

In the chicken, B genotype is the most influential determinant of tumor outcome. Collins et al. (1977) studied F_2 segregants of a cross between RPRL lines 6_1 and 15_1. The percentage of chicks which died of terminal tumors, by haplotype, was: B^5B^5, 93%; B^2B^5, 26%; B^2B^2, 5%. Schierman, Watanabe and McBride (1977), in an independent study, defined the dominant R-Rs-1 gene, which determined the regressor phenotype, and was associated with the B^2 genotype. The recessive allele for progressive tumor growth was designated r-Rs-1, and was associated with the B_1 haplotype. It is unknown at present whether identical B designations in the two studies denote the same genes.

IMMUNITY TO RSV-INDUCED TUMORS

Immunity to RSV-induced tumors was assayed in pure progressor or regressor line chickens by administering a challenge to the right wing at specific intervals after the first inoculation in the left wing (Gyles, Blythe, Test, Bowanko and Brown, 1977). B haplotypes of the two lines were not reported. While immunity developed earlier in the regressor line, by 15 days Post Inoculation (PI), chickens of both lines

resisted a second challenge of either virus or tumor homogenate. McBride, Watanabe, and Schierman (1977) extended these findings to their chicken line (GB-1) which carries the r-Rs-1 (tumor progression) gene. In this case, all GB-1 chickens tested were resistant to a second virus-challenge 8 days following primary tumor induction. An unexpected finding was that the second inoculation of RSV resulted in a marked increase in growth rate of the primary tumor. This enhanced rate of growth required an intact Bursa of Fabricius, even though bursectomy did not influence immunity to the secondary challenge.

T-CELL FUNCTION AND MITOGEN REACTIVITY

The plant lectins phytohemagglutinin (PHA) and concanavalin A (con A) are specifically mitogenic for T lymphocytes. When cultured *in vitro* with optimum proportions of either con A or PHA, murine T-cells, but not B cells, were stimulated to synthesize RNA and DNA, and then to undergo mitosis (Janossy and Greaves, 1972). Most procedures in current use are derived from the microtest system of Park and Good (1972), which compared the incorporation of radiolabeled precursors of DNA into cells cultured with and without mitogen.

Specificity of PHA and con A for chicken T-cells was demonstrated by Greaves, Roitt and Rose (1968) and Toivanen and Toivanen (1973), respectively. Genetic control of stimulation by con A was examined by Miggiano, North, Buder and Pink (1976), who showed a five-fold difference in response by lymphocytes from different inbred lines of chickens. The

genetic locus responsible for this difference (designated Mrl) was not linked to the MHC.

MITOGEN STIMULATION AS A MEASURE OF IMMUNE REACTIVITY IN CANCEROUS HOSTS

Mitogen stimulation of human peripheral lymphocytes was recommended as a means of testing over-all T-cell function (Fudenberg, Good, Hitzig, Kunkel, Roitt, Rosen, Rowe, Seligmann and Scothill, 1971). Responses to PHA or con A, when included in immunoprofile studies of cancer patients, have given equivocal results, possibly due to the genetic heterogeneity of the human population (Cochran, Mackie, Grant, Ross, Connell, Sandilands, Whaley, Hoyle and Jackson, 1976; Concannon, Dalbow, Eng and Conway, 1977). In one study (Dalbow, Concannon, Eng, Weil, Conway and Nambisan, 1977), statistically significant correlations were observed between the responses of lymphocytes, the stage of disease and the period of survival. However, the correlated responses were of limited prognostic value for individual patients. Interestingly, the response of lymphocytes of cancer patients to PHA was improved by washing the cells (Mannick, Constantine, Pardridge, Saporoschetz and Badger, 1977).

Marek's disease of chickens has been associated with a depression of both thymus- and bursa- dependent immune systems (Burg, Feldbush, Morris and Maag, 1971). Animals with Marek's tumors had lower levels of stimulation for both con A (Lu and Lapen, 1974) and PHA (Theis, McBride and Schierman, 1975), than did control birds. Schat, Schultz and

Calnek (1977) found a transient depression of response to con A at 7 days post inoculation (PI) with both oncogenic (GA-5) and non-oncogenic (SB-1) variants of virus. A permanent depression, which started 2 weeks PI, developed only in genetically susceptible chickens inoculated with GA-5.

CELLULAR STAINING WITH ACRIDINE ORANGE

Acridine orange (AO) is a metachromatic dye which exists in monomeric form at low concentrations in solution, and fluoresces green when excited with ultraviolet or blue light. At higher concentrations, AO dimerizes and fluoresces red (Bradley and Wolf, 1959). Under appropriate conditions, AO preferentially stains nucleic acids among other cellular polyanions (Darzynkiewicz, Traganos, Sharpless and Melamed, 1976). The dye intercalates into the DNA double helix as a monomer which fluoresces green (Lerman, 1963), while stacking via electrostatic interaction on phosphates of single-stranded nucleic acid, resulting in dye interactions and red fluorescence (Bradley and Wolf, 1959). Differentiation of DNA and RNA by AO staining requires prior denaturation of double stranded RNA (Darzynkiewicz et al., 1976) which can be accomplished by the addition of EDTA (Braunstein, Melamed, Darzynkiewicz, Traganos, Sharpless and Good, 1975).

ACRIDINE ORANGE AND AUTOMATED CYTOPHOTOMETRY

A flow cytophotometer is an instrument which measures light emitted from individual cells passing through a flow

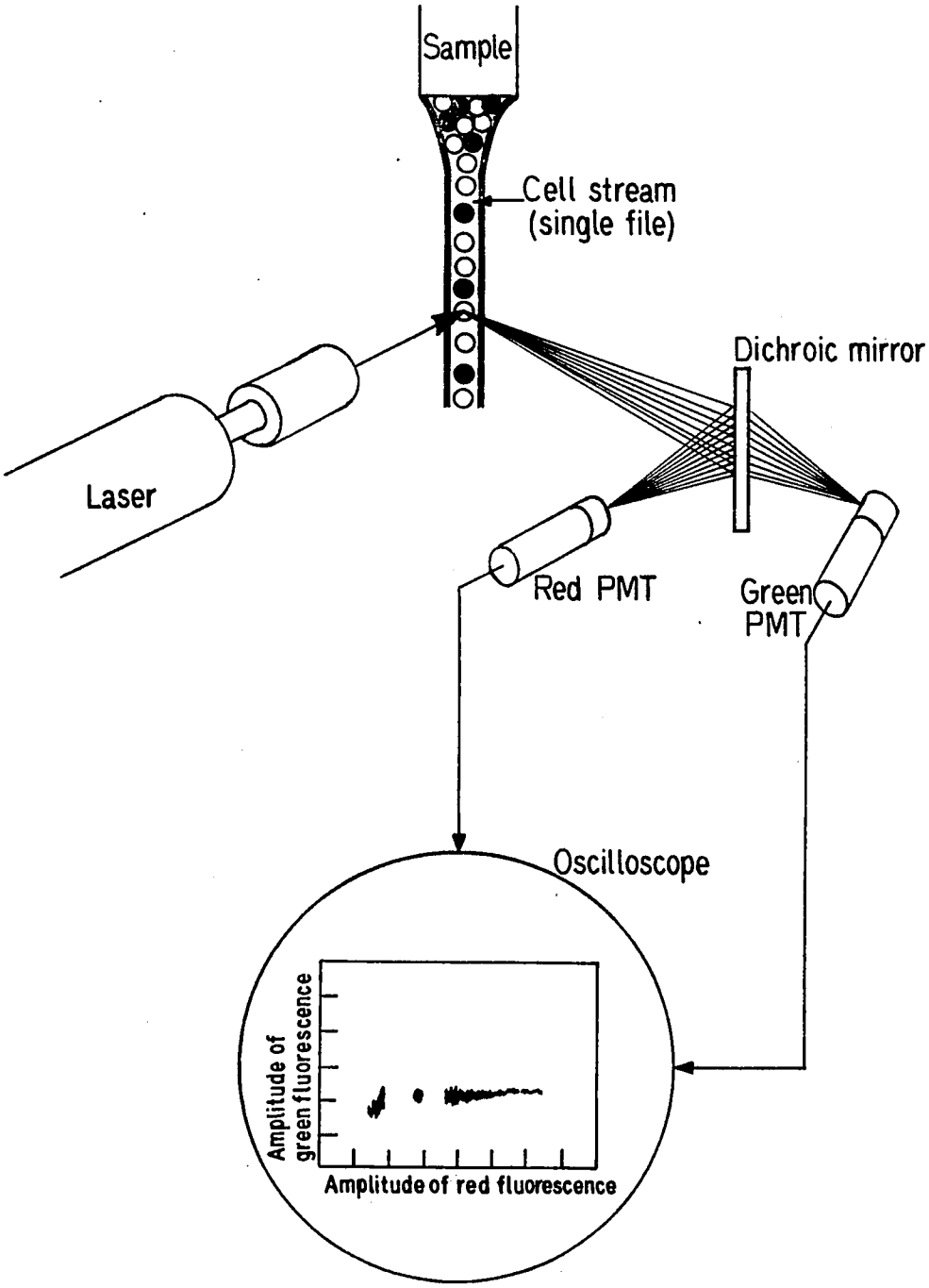
chamber, usually at a rate of 200 to 1000 cells per second. In the application of AO to automated cytophotometry, cells flow past a 488 nm argon ion laser (Fig. 1) and emit red and green fluorescence which is measured by separate photomultiplier tubes. A multichannel distribution analyzer is then used to plot a point representing each cell on a histogram displaying the intensity of red and green fluorescence. Therefore, tens of thousands of cells can be individually analyzed for content of DNA and RNA in a short time.

AO staining plus automated cytophotometry has been used for automated differential counting of human leukocytes (Adams and Kamentsky, 1974), for detection of changes in granulocytes with infection (Melamed, Adams, Traganos and Kamentsky, 1974) and for quantitation of PHA transformed lymphocytes (Braunstein et al., 1975). Darzynkiewicz et al. (1976) used a Cytofluorograf(R) to follow the progression of PHA-stimulated human lymphocytes through the stages of the cell cycle. In pure lymphocyte preparations, they were able to differentiate 5 categories of cells: dead, G_0, G_1, S and G_2+M. Using nucleases, they also determined that at least 88% of green fluorescence was due to DNA and at least 87% of red fluorescence was due to RNA.

ACRIDINE ORANGE STAINING IN CANCER DIAGNOSIS

AO staining for cytodiagnosis of cancer was first applied to exfoliative gynaecology and malignancies of the respiratory system (von Bertalanffy and Beckis, 1956). Currently, an automated prescreening program is in use, which

Fig. 1. Diagrammatic representation of a flow cytophotometer. Cells stained with acridine orange are excited by a 488-nm argon ion laser, and red and green fluorescence is quantitated by separate photomultiplier tubes. Each cell is represented on the oscilloscope as a point, with intensity of green fluorescence on the ordinate, and of red fluorescence on the abscissa.


Sample
Cell stream
(single file)
Dichroic mirror
Laser
Red PMT
Green
PMT
Oscilloscope
Amplitude of
green fluorescence
Amplitude of red fluorescence

indicates diagnosis by AO staining is applicable to the entire spectrum of cellular abnormality occurring in the human female genital tract (Wheeless, Hardy and Balasubramanian, 1975).

Cucchiara (1976) correlated red fluorescence of AO stained peripheral lymphocytes with RSV-induced tumor development in chickens. He observed by microscopic examination that red-staining peripheral cells increased from 0.5% before inoculation to about 24% when tumors were maximal. Neither lymphocytes from uninoculated controls nor from chickens inoculated with three non-oncogenic RNA viruses exhibited red fluorescence.

RATIONALE FOR THE PRESENT STUDY

While many parameters are known to correlate with tumor progression or regression, clearly defined cause and effect relationships remain elusive. Since a finite group of linked genes (MHC) on a single chromosome can determine survival or death of the tumor-bearing host, identifiable phenotypic differences should exist. The present study was undertaken to explore previously reported lymphocyte-associated characteristics, in a system where the progression or regression of tumors was predetermined by MHC genotype. It is hoped that the results reported herein will add in part to the understanding of a very complex disease.

CHAPTER III

MATERIALS AND METHODS

CHICKEN STOCKS

The single-comb white leghorn chickens used in this study were developed at the Regional Poultry Research Laboratory (RPRL), East Lansing, Michigan (Waters, 1940). Line 6 subline 3 (6_3), homozygous B^2 animals were obtained as day-old chicks from RPRL. The University of New Hampshire (UNH) poultry farm supplied fertile eggs from RPRL lines 6_1 and 6_3 (homozygous B_2), RPRL line 15_1 (homozygous B^5), and F_2 and F_3 progeny selected for B genotype. All chickens obtained from the UNH poultry farm were typed at the B-blood group locus by Dr. W. E. Briles, Northern Illinois University, DeKalb, Ill.

BROODING AND HATCHING

Hatching-eggs from the UNH poultry farm were fumigated for 1 h in formaldehyde vapors, cleaned with 95% ethanol, and incubated at 37 C until hatching. All chicks were brooded for 3 to 6 weeks in conventional, electrically heated, thermostatically controlled brooding batteries in an isolated animal facility. Wherever possible, aseptic procedures were employed to keep chicks free from endemic diseases. Throughout the study, animals were fed medicated Merrimack Chick Starter (Merrimack Farmers Exchange, Concord, NH 03301) ad libitum.

VIRUS STOCK AND INOCULATION

Bryan high-titer strain Rous sarcoma virus (RSV) in combination with the helper virus, Rous associated virus (RAV-1), was obtained from Dr. L. B. Crittendon (RPRL), and stored in liquid nitrogen. The stock virus (designated RSV-1; for a review of nomenclature, see Hanafusa, 1977) was diluted in Hanks balanced salts solution (see appendix) containing 5% fetal calf serum plus 100 units penicillin, 100 ug streptomycin (GIBCO) and 10 ug hyaluronidase (Sigma Chemical) per ml. Six-week old birds were injected subcutaneously in the left wing-web with 0.1 ml of a 10^{-3} dilution (approximately 20 pox-forming units) of RSV-1 unless otherwise indicated. Tumors appeared in 7 to 10 days and tumor size was scored subjectively according to Collins *et al.* (1977).

BLOOD COLLECTION AND LEUKOCYTE SEPARATION

Blood samples were obtained by either cardiac or brachial vein puncture. Blood was collected aseptically into sterile heparinized (50 units/ml whole blood) syringes or vials and used in the following procedures to isolate lymphocytes:

a) Slow centrifugation (adapted from Jones, 1977). Ten ml of blood were centrifuged in a 12 ml conical centrifuge tube for 10 min, 90 x g, at room temperature, in a swinging bucket centrifuge (International Model UV centrifuge with Model 240 rotor). The buffy coat was carefully stirred

into the plasma layer, and the lymphocyte rich plasma was removed with a sterile pasteur pipette and placed in a 14x10 mm centrifuge tube.

b) Ficoll-Diatrizoate (FD) centrifugation (a modification of the procedure of Archambault et al., 1976).

Two ml of blood were carefully layered over 2 ml of FD in a 14x100 mm serological tube. FD consisted of 9 percent Ficoll (Sigma) and 33.9 percent sodium diatrizoate (Winthrop) with a specific gravity of 1.085 to 1.090 (see appendix). Blood layered over FD was centrifuged at 800 x g for 6 to 8 min in a swinging bucket centrifuge. The lymphocyte-rich layer at the interface between FD and plasma was removed with plasma (but no FD) and placed in a 14x100 mm centrifuge tube.

DIFFERENTIAL AND VIABILITY COUNTS

Differential counts of separated leukocytes were made by counting a minimum of 100 cells according to Natt and Herrick (1954). Briefly, cells were diluted (1:10 or 1:100) in Natt-Herrick stain (appendix), left at room temperature for 2 min, then loaded into a hemacytometer (American Optical) and counted under 400X magnification.

Viable counts were made according to Hudson and Hay (1976). Cells were diluted in 1.11X (according to Gilmour, 1976) Dulbecco's phosphate buffered saline (PBS) containing 0.2% nigrosin (MC/B), left at room temperature for 5 min, and

counted under 400X magnification in a hemacytometer. Refractile cells were counted as viable while dark cells (containing stain) were considered dead.

ACRIDINE ORANGE (AO) STAINING

Peripheral blood leukocytes were separated by centrifugation on FD (procedure above), pelleted at 400 x g for 10 min and resuspended in Dulbecco's 1.11x PBS. Initially, cells were fixed and stained according to Cucchiara (1976); however, this procedure was unsatisfactory due to extensive lysis and aggregation of cells, rapid fading of red fluorescence, and loss of greater than 90% of cells during fixation. Consequently, the method of Darzynkiewicz et al. (1976) was used. Cells (optimally 2 to 5 X 10^5), in 0.2 ml of serum or PBS, were added to 0.5 ml of a solution containing 0.1% Triton X-100 (Packard), 0.2 M sucrose, 0.0001 M EDTA, and 0.02 M citrate-phosphate buffer (pH 3.0). After 1 min, 1 ml of staining solution (0.002% AO, 0.1 M NaCl, 0.01 M citrate-phosphate buffer, pH 3.8) was added. Staining was for 5 min. A wet mount was prepared and cells were observed with a Reichert fluorescence microscope (exciting filter #KG-2/BG-12, barrier filter #1.5/OG 1-1/BG-9).

Automated cytophotometric analysis (described earlier) required cells to be fixed and stained at UNH, and shipped to Los Alamos, New Mexico, for analysis. This required that the AO-staining characteristics be stable at 5-10 C for up to 48 h. The glutaraldehyde post-fixation method of Cambier, Wheeless and Patten (1977) caused extensive clumping of cells,

possibly leading to red-staining artifacts (Darzynkiewicz et al. 1975). Several variations of Cambier et al.'s procedure were tried, and the following method was developed.

Leukocytes were separated on FD, harvested with autologus serum, and diluted with 1.11X Dulbecco's PBS to a cell density of 2.5 X 10^7/ml. Two-tenths ml of cell suspension was added to 0.5 ml of cold phosphate buffered (0.1 M, pH 6.0, see appendix) 3.8% glutaraldehyde. Fixed cells were shaken for 15 sec and 1 ml of freshly prepared AO stain (30 ug AO/ml in 0.1 M citrate, 0.2 M phosphate buffer, pH 6.0) was added. Cells were stained 15 min at room temperature in the dark, centrifuged at 150 X g for 10 min, resuspended in 5 ml phosphate buffered glutaraldehyde, and stored at 5 C in the dark until analyzed.

CYTOPHOTOMETRIC ANALYSIS

Initially, FD-separated leukocytes were fixed in 70% methanol-30% acetone, and shipped to Dr. Z. Darzynkiewicz (Memorial-Sloan Kettering Cancer Center, New York City) for AO staining and analysis in a Systems 40 Cytofluorograf (Ortho Instruments). Later, glutaraldehyde fixed, AO stained cells were shipped to Dr. J. Leary (Los Alamos Scientific Laboratory, New Mexico) for analysis in the Los Alamos Cell Sorter as described. Cells were shipped and stored at 5 C in the dark up to 72 h before analysis.

MITOGEN ASSAYS

Chickens with regressor (B^2B^2) and progressor (B^5B^5) haplotypes were tested for the response of their lymphocytes to the T-cell mitogens concanavalin-A (con A) and phytohemagglutinin (PHA). Lymphocytes were isolated by slow centrifugation, washed twice with Dulbecco's 1.11x PBS and resuspended in RPMI 1640 culture medium (GIBCO) containing 2 mM L-glutamine (freshly added), 10% fetal calf serum (FCS), 100 ug streptomycin and 100 units penicillin (pen-strep) per ml (this media is designated 1640+). Cell density was adjusted to 1.33×10^7 cells/ml and 150 ul were added to each of nine microtiter wells (Vanguard, U-bottom plate #1482) for each lymphocyte sample tested. Either 10 ug con A (Miles) or 80 ug PHA (Sigma) were added to each of 3 test wells in 50 ul of 1640+. Fifty microliters of 1640+ were added to 3 control wells. Cultures were incubated for 72 h in a humidified 5% CO_2 incubator at 37 C, cultured for 16 h with 1 uCi tritiated thymidine (New England Nuclear, 5.7 Ci/m mole) and harvested by suction onto glass fiber filters (Whatman #934) using a Mash II (Microbiological Associates) cell harvester. Filters were dried at 80 C for 1 h, placed in scintillation vials with 10 ml toluene (Baker) containing 0.4% 2,5 diphenyloxazole (PPO) and 0.00525% 1,4-bis /2-(5-phenoxazolyl)/-benzene (POPOP) (Packard) and counted in a Packard Tri-Carb scintillation counter (gain 51%, windows 50-1000). A stimulatory index (SI) was determined for each sample by dividing the mean cpm of mitogen treated cells by the mean cpm of untreated cells.

PROTEIN ELECTROPHORESIS

Serum proteins were analyzed by electrophoresis in discontinuous polyacrylamide gels containing sodium dodecyl sulfate (SDS). A model SE 500 slab gel apparatus with model PS101 power source (Hoefer) was used. Formulae (modified from Laemmli, 1970) were as follows:

1) A 12 cm long, 0.7 mm thick separating gel consisted of 7.0% acrylamide (Ames), 0.025% N,N,N',N'-Tetramethyl-ethylenadiamine (Temed;Kodak), 0.1% SDS (Matheson), and 0.025% ammonium persulfate (Kodak) in 0.375M Tris (hydroxymethyl) aminomethane (Tris-HCl; Sigma) buffer at pH 8.8.
2) A 1 cm long stacking gel was 3.0% acrylamide, 0.045% Temed, 0.1% SDS, and 0.045% ammonium persulfate in 0.125M Tris-HCl at pH 6.8.
3) Samples contained from 5 to 100 ug protein in 0.0625 M Tris-HCl (pH 6.8) containing 2.0% SDS, 10.0% glycerol (Fisher) 5.0% 2-mercaptoethanol (Sigma) and 0.01% bromphenol blue (Allied).
4) Electrode buffer was 0.025 M Tris, 0.192 M glycine (Sigma) and 0.1% SDS. Ten or 20 ul of sample were added to each well of the slab, and electrophoresed at 12 mA for 45 min, then at 20 mA until the tracking dye migrated 12 cm (approximately 3 h).

Serum samples were collected according to Cucchiara (1976).

CHAPTER IV

RESULTS

EFFECT OF HOST GENOTYPE AND AGE ON TUMOR DEVELOPMENT

Originally, line 6_3 chickens were used to compare leukocytes (by acridine orange staining) and serum (by gel electrophoresis) from tumor-bearing and non tumor-bearing chickens. Table 1 shows that maximum tumor was obtained in only 4 of 105 chickens from this line; three cases occurring during an epidemic of aplastic anemia in the flock, and thus of questionable value. Aseptic procedures, isolation, and use of specific pathogen-free (SPF) birds from RPRL resulted in even fewer tumors of maximum size (Table 1, RG18H1 through H4). Consequently, F_2 and F_3 progeny of crosses between lines 15_1 and 6_3 or 6_1 were used. Table 2 shows that the present results concurred with those of Collins et al. (1977); birds with the genotype B^2B^2 usually developed tumors which regressed, while B^5B^5 birds invariably died with a large tumor. Moreover, there was no apparent difference between the B^2 allele of lines 6_1 and 6_3 as it affected tumor regression. Nor did the source of chicks (RPRL or UNH) affect TPI's.

The TPI of B^5B^5 birds (6_1 x 15_1, F_3) was consistent when inoculated between 6 and 8.5 weeks of age (Table 3). B^2B^2 chickens, when challenged at the older age, may have regressed their tumors more quickly.

Table 1. Distribution of Line 6_3 Chickens According to Tumor Profile Index (TPI), and Attainment of Maximum Tumor Size, by Hatches.

Hatch	Age At Inoculation (Weeks)	TPI[1] 1	2	3	4	5	Maximum[2] Tumor	Total
RMZ H_4 UNH Line 6_3	6	3	3	6	0	6[3]	1[3]	18
RMZ H_5 UNH Line 6_3	6	0	5	9	0	4[3]	2[3]	18
RG18 H_1 RPRL Line 6_3	6	0	2	5	0	1	1	8
RG18 H_2 RPRL Line 6_3	7	4	14	14	0	0	0	32
RG18 H_3 RPRL Line 6_3	6	1	5	4	0	0	0	10
RG18 H_4 RPRL Line 6_3	6	7	9	3	0	0	0	19
TOTAL		15	38	41	0	11	4	105

[1]Tumor profile indexes (TPI's) according to Collins et al. (1977), TPI 1-3 = Regression before 70 days; TPI 4 = tumor palpable at 70 days; TPI 5 = death due to tumor prior to 70 days.

[2]Number of birds with a maximum tumor score size of 6, according to Collins et al., 1977.

[3]TPI's and maximum tumor size may be elevated due to epidemic of aplastic anemia during these experiments.

Table 2. Distribution of F_2 Generation Progeny According to Genotype and TPI, Combined Data from Three Hatches per Mating.

	PARENTAL LINES					
	RPRL (6_3 x 15_1)			UNH (6_1 x 15_1)		
	B Genotype			B Genotype		
TPI[1]	22	25	55	22	25	55
1	4	0	1	12	0	0
2	6	8	0	19	3	0
3	3	18	2	4	2	0
4	0	3	0	0	2	0
5	1	1	24	1	1	18
TOTAL	14	30	27	36	8	18

[1]For TPI Designations, See Table 1.

Table 3. Distribution of 6_1 x 15_1 (F_3) Chickens According to Tumor Profile Index (TPI), Attainment of Maximum Tumor Size, and Age at Virus Inoculation, by Hatches.

Hatch	Age Inoculation (Weeks)	B-Genotype	TPI[1] 1	2	3	4	5	Maximum Tumor[2]	Total
$RG21H_5W$	6	55	0	0	0	0	9	9	9
		22	1	6	4	0	0	0	11
$RG21H_5$	6	55	0	0	0	1	11	11	12
		22	7	2	4	2	0	0	15
$RG21H_4W$	7	55	0	0	0	0	7	5	7
		22	0	6	0	0	0	0	6
$RG21H_1W$	7.5	55	0	0	0	0	5	5	5
		22	1	6	3	0	0	0	10
$RG21H_3W$	8	55	0	0	0	0	5	5	5
		22	4	1	1	0	1	1	7
$RG21H_{10}W$	8.5	55	0	0	0	0	10	9	10
		22	8	6	0	0	0	0	14

[1]For TPI Designations, See Table 1.

[2]Number of birds with a maximum tumor score of 6_1 according to Collins et al., 1977.

LYMPHOCYTE PURIFICATION FROM BLOOD SAMPLES; A COMPARISON OF METHODS

Lymphocytes were isolated according to Archambault et al. (1976), Lee (1974), Jones (1977), and Miggiano et al. (1976). A modification of the procedure of Archambault et al. (centrifugation through FD) resulted in greater than 90% recovery of lymphocytes from blood, but thrombocyte contamination often equalled lymphocyte recovery. Conversely, the procedure of Jones (slow centrifugation) recovered about 10% of blood lymphocytes, with few contaminating cells. The other methods were unsatisfactory.

Table 4 shows recovery and purity for 6 blood samples which were split, and the leukocytes separated by both FD and slow centrifugation. Note that FD recovered 7.7 times as many cells as slow centrifugation, but only 60% of these cells were lymphocytes. Leukocyte purification was unsuccessful in about 15% of chickens with large tumors (e.g. sample #NB in Table 1). Leukocytes were purified from a total of 47 samples by slow centrifugation alone, with a mean recovery of 4.06×10^6 cells/ml of blood. Isolated leukocytes consisted of greater than 90% lymphocytes, 0.5-9% erythrocytes, and less than 1% thrombocytes or granulocytes.

Seventy-one samples purified by FD centrifugation had a mean recovery of 2.92×10^7 cells/ml, with 23.2-88.5% lymphocytes, 0.5 to 30.7% erythrocytes, 3.4 to 79.4% thrombocytes, and 0.5 to 26.2% other leukocytes. The respective means were

Table 4. Cell Recovery from Blood by Ficoll-Diatrizoate (FD) or Slow (S) Centrifugation.

	Cell Yield Per ml Blood		Percent Lymphocytes		Percent Erythrocytes		Percent Thrombocytes		Percent Granulocytes[2]	
Sample #	FD	S	FD	S	FD	S	FD	S	FD	S
7003	1.7×10^6	1.7×10^6	63	93.4	10.4	0.6	16.8	5.4	9.8	0.6
7108	4.0×10^6	4.3×10^6	62.7	91.5	1.3	3.2	33.6	3.9	2.5	1.4
7187	2.7×10^7	6.5×10^6	60.1	88.2	1.3	4.4	33.3	4.4	5.3	3.0
7196	4.1×10^7	1.4×10^6	50.8	88.9	2.3	2.7	44.4	8.1	2.5	0.3
7201	3.0×10^7	2.2×10^6	61.0	94.9	10.2	0	20.3	2.9	8.5	2.2
NB[1]	1.2×10^8	2.9×10^6	15.0	32.1	63.3	57.4	10.6	7.0	11.1	3.5
Mean	2.1×10^7	3.2×10^6	59.5	91.4	5.1	2.2	29.7	4.9	5.7	1.5
Standard Deviation	1.7×10^7	2.2×10^6	5.02	2.86	4.77	1.84	11.17	1.98	3.36	1.12

[1]Animal with apparent blood dyscrasia, not included in mean and standard deviation.

[2]Includes unidentifiable cells.

53.2%, 7.7%, 33% and 6.6%. Cells were consistently greater than 90% viable for both methods of separation.

ANALYSIS OF LYMPHOCYTES BY ACRIDINE ORANGE STAINING

A COMPARISON OF MICROSCOPIC METHODS

Lymphocytes were originally stained and the percentage of FD-separated leukocytes having intense red fluorescence was determined according to the method of Cucchiara (1976). One hundred seven samples from uninoculated controls, 18 from animals with maximum wing tumors, and 9 from chickens with smaller wing tumors were analyzed. Mean percentages of intensely red fluorescing cells, $\pm$ standard deviations were 6.23 ± 7.67, 29.03 ± 22.36 and 14.1 ± 23.32, respectively. Thus great variability among samples and poor correlation of the incidence of red fluorescing cells with tumor size was observed, contrary to the results of Cucchiara (1976). Examination of additional samples showed that this procedure eliminated greater than 90% of cells from analysis, caused extensive lysis, and was accompanied by rapid fading of red fluorescence. Moreover, contaminating polymorphonuclear cells, if any, could not be differentiated from mononuclear cells.

Due to the unacceptable variability of the above procedure, the method of Darzynkiewicz *et al.* (1976) was used for all remaining fluorescence microscopy. Since cells were stained without fixation and in suspension by this method, no cell loss or fixation artifacts were encountered. Nuclear integrity was maintained, and cell types could be distinguished

microscopically (Fig. 2). However, fading of red fluorescence still occurred within 15 to 30 min.

TUMOR ASSOCIATED INCREASE IN RED FLUORESCING LEUKOCYTES DETECTED BY AO STAINING ACCORDING TO DARZYNKIEWICZ.

Tumor-bearing chickens had significantly ($P < .05$) more red fluorescing cells than controls between days 21 and 29 post inoculation (Fig. 3). Nine observations on positive controls (chickens with maximum wing tumors) had a mean of 22.77% intensely red cells and a standard deviation of 8.17. Thus the staining method of Darzynkiewicz confirmed the increased red fluorescence of lymphocytes from tumor-bearing hosts which had been observed by Cucchiara (1976). However, these results differed from the former study in that increased red fluorescence accompanied tumor growth, rather than preceding it, and great variation existed among control samples.

Although this staining procedure was an improvement, the subjectivity of manual counting still resulted in unacceptable variation. For example, counts of red-fluorescing cells in a single sample by two technicians differed as much as six-fold. Also, about 5% of samples had inexplicably high or low counts, and in a second study dealing with the appearance of red fluorescing peripheral lymphocytes ($RG18H_2$), there was no significant difference between red-cell counts of tumorous and control animals.

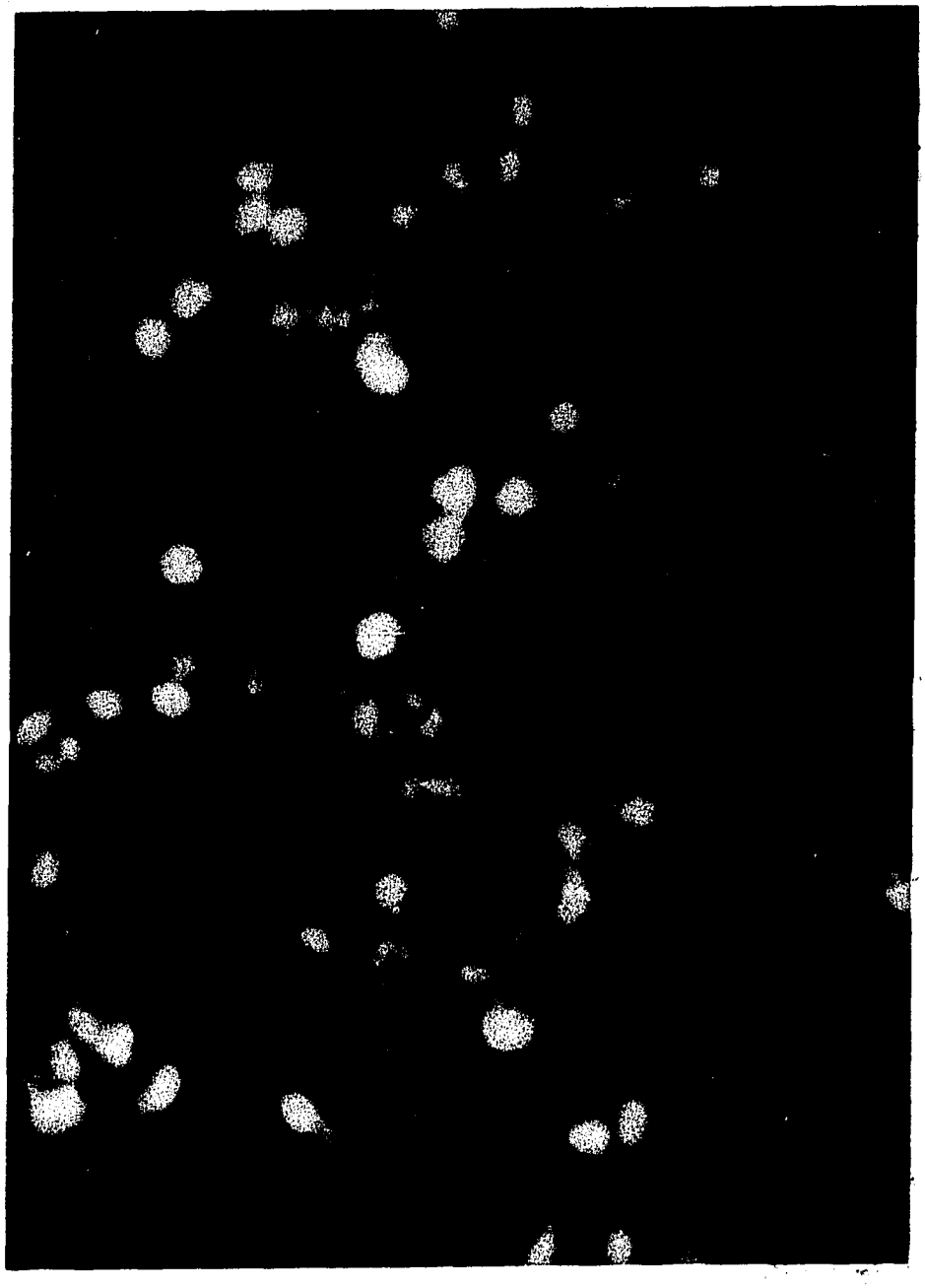


Fig. 2a. Photomicrographs of FD-separated cells after staining with acridine orange according to Cucchiara (1976). (X2800)

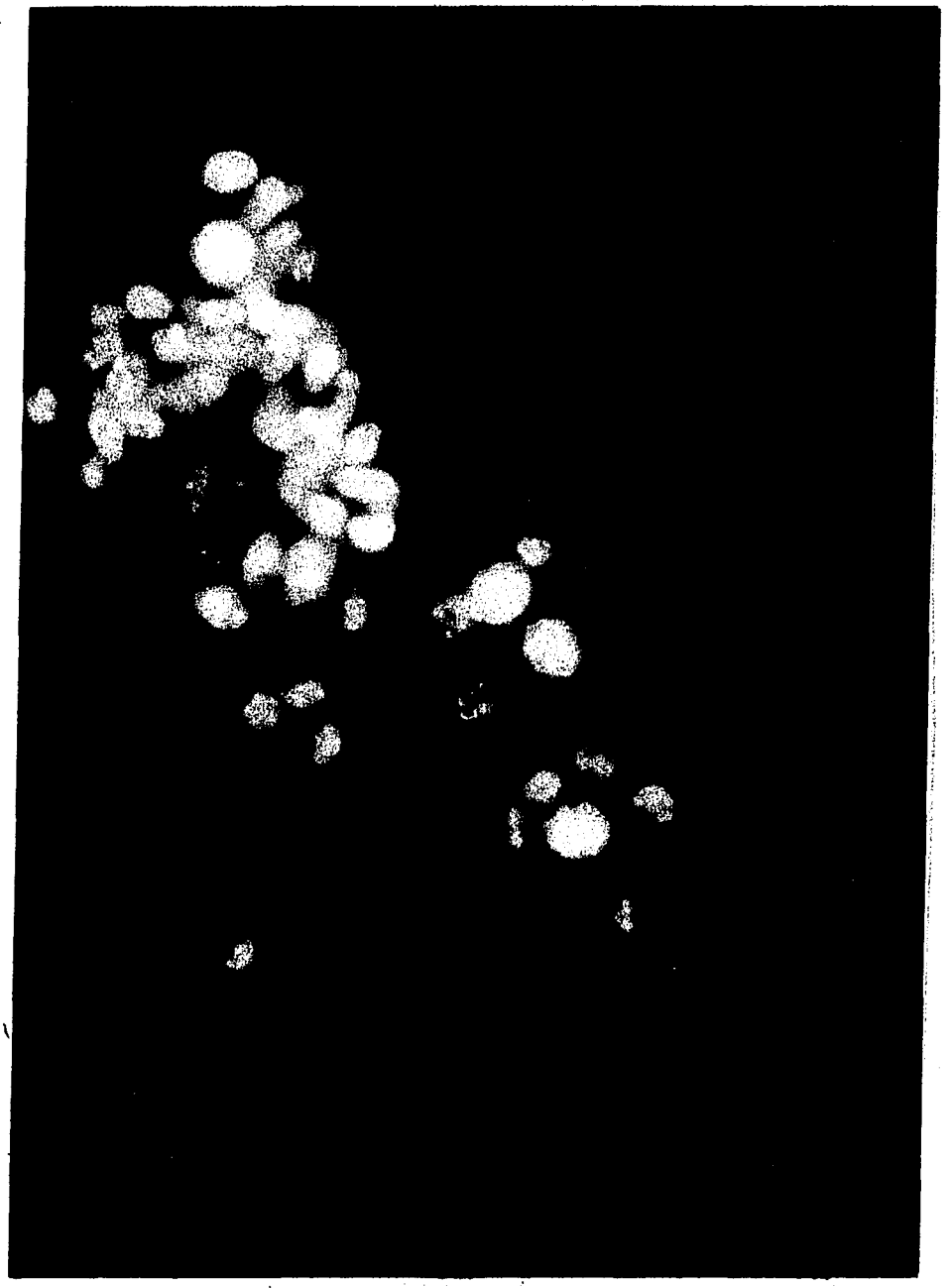
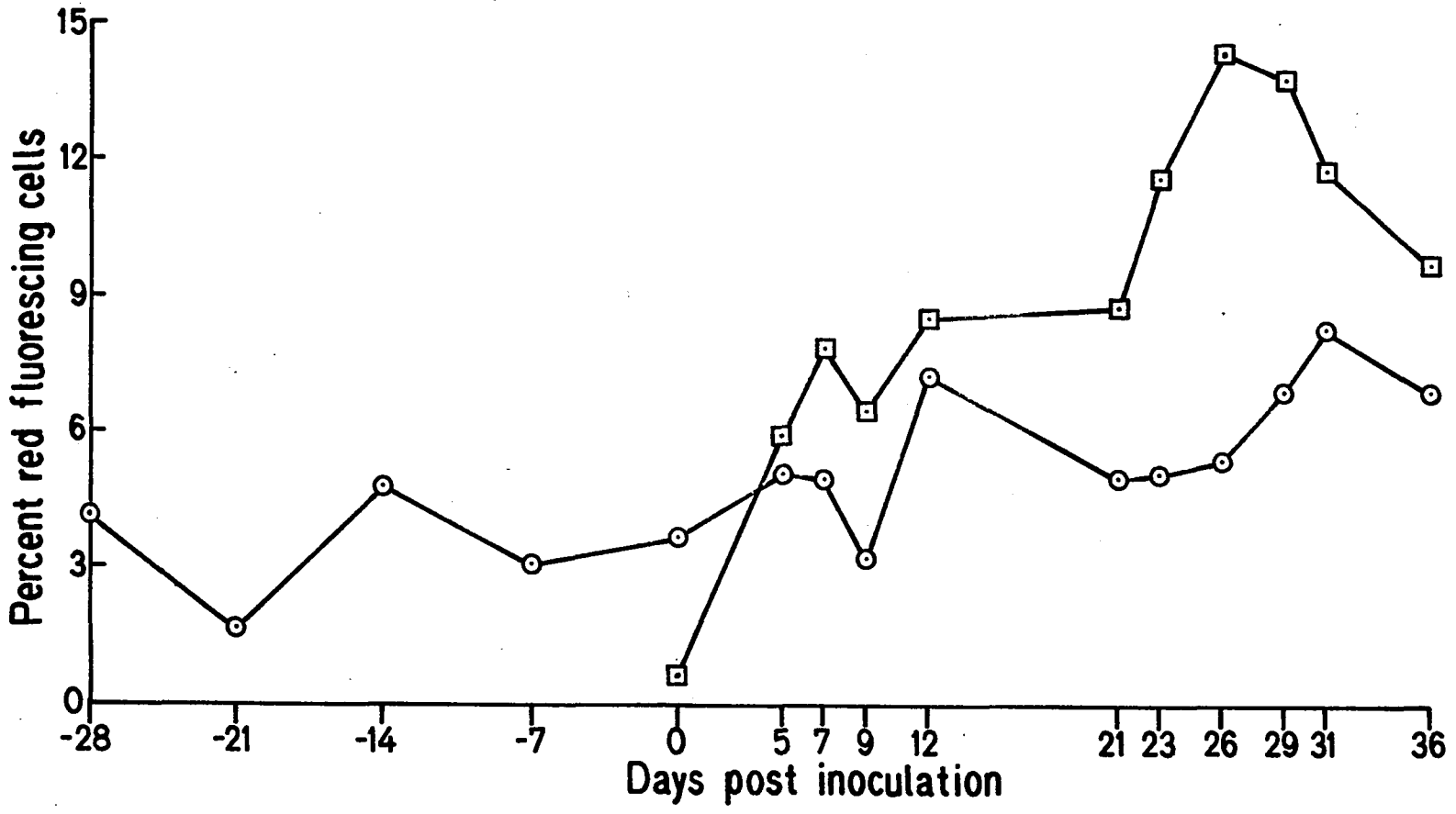


Fig. 2b. Photomicrographs of FD-separated cells after staining with acridine orange according to Darzynkiewicz et al.(1976) (X2800)

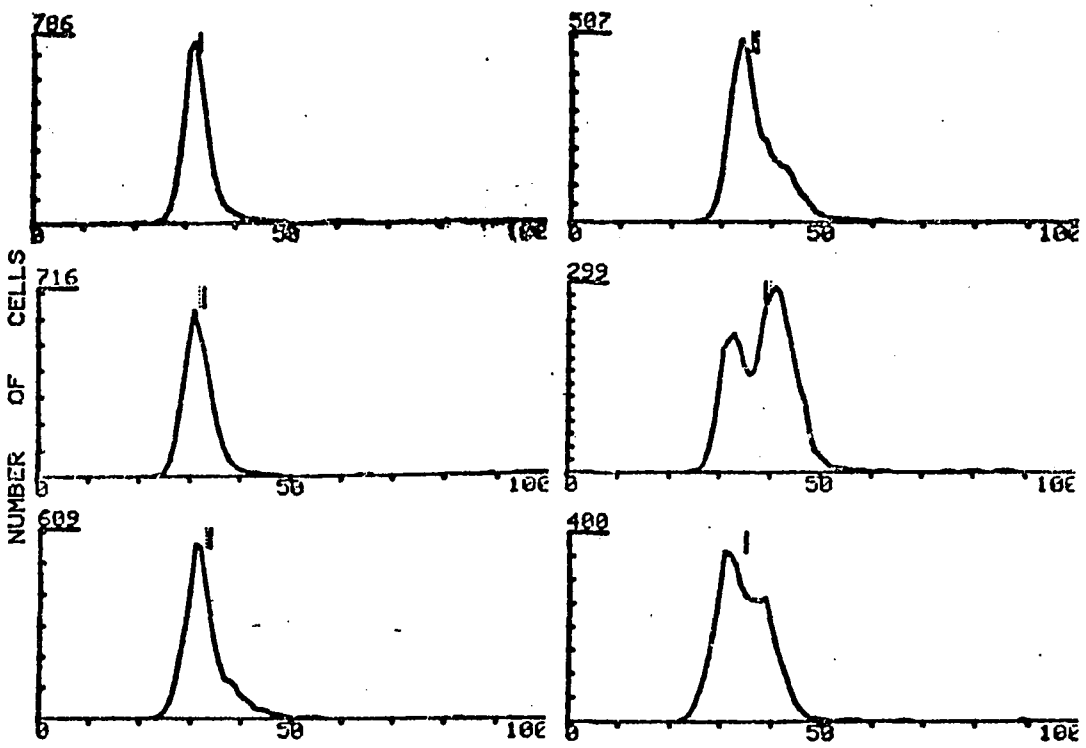
Fig. 3. Mean percentage of red fluorescing cells after staining with acridine orange according to Darzynkiewicz (1976); inoculated (⊡) and control (⊙) chickens.


Percent red fluorescing cells
15
12
9
6
3
0
-28
-21
-14
-7
0
5
7
9
12
21
23
26
29
31
36
Days post inoculation

TUMOR-RELATED INCREASE IN LEUKOCYTES WITH ELEVATED RED FLUORESCENCE AS CONFIRMED BY AUTOMATED CYTOPHOTOMETRY.

To alleviate the problems of manual counting, ten samples were analyzed in a cytofluorograph(R) automated cell cytophotometer by Dr. Z. Darzynkiewicz. Cells were isolated from cardiac blood by centrifugation on FD, added in autologous plasma to 3 ml of 1.11x PBS containing 2mM $MgCl_2$, and centrifuged at 400xg for 10 min. Pellets were resuspended in 2 ml of 1.11x PBS, and split into 2 aliquots, one of which was added to 9 ml of 50% acetone, 50% ethanol. Aliquants of these fixed cells were shipped in 1-dram vials (Wheaton) at 10 C for analysis. At the laboratory of Dr. Darzynkiewicz, the samples were centrifuged, resuspended in 0.15N NaCl containing 0.05 N HCl, and stained with citrate (0.1M) - phosphate (0.2M) buffered (pH 6.0) saline (0.15 M NaCl) containing 6 ug/ml acridine orange. Ten thousand cells per sample were individually assayed for intensity of green or red fluorescence, and a frequency distribution was generated with a multi-channel analyzer. Fig. 4 shows typical distributions for red fluorescence of cells from three control chickens and three animals with maximal tumors. Few cells from controls, but many from tumor-bearing chickens had an intensity of red fluorescence greater than 40, based upon a relative scale from 0 to 100. To determine whether or not cells with elevated red fluorescence could attach to a substrate, $1x10^7$ cells from each of 6 samples were suspended in 20 ml of BME culture medium (GIBCO) + 10% FCS in a culture dish (Falcon #3007) and incubated in a 5% CO_2 incubator at 37 C for 30 min. Non-adherent cells were

Fig. 4. Frequency distributions for red fluorescing leukocytes from tumor-bearing and uninoculated hosts.


786
507
716
292
602
480
0
50
100
NUMBER OF CELLS
INTENSITY OF RED FLUORESCENCE
(UNINOCULATED CONTROLS)
INTENSITY OF RED FLUORESCENCE
(TUMOR-BEARING BIRDS)

transferred to a second dish and incubation continued for an additional 30 min. After monolayering, cells remaining in suspension were washed, fixed, and shipped to Dr. Darzynkiewicz' laboratory for analysis, as described above.

Table 5, a summary of cytophotometric analysis, shows elevated red fluorescence in animals with large, progressing tumors when compared with regressors or controls. Host number 10, which had a massive tumor when sampled, did not have cells with elevated red fluorescence, and its tumor completely regressed. Table 5 also shows that monolayering removed less than half of the cells with elevated red fluorescence for both controls and tumorous chickens. Since cells in the second dish of the monolayering procedure were not near confluency, it is presumed that remaining cells were unable to attach to substrate, and were probably lymphoid cells. The Pearson correlation coefficient for tumor progression (set to equal 1) or regression/control (set to equal 2) versus automated counts of red fluorescence was .966; for manual counts versus progression or regression/control was .793; and for manual versus automated counts was .772. Therefore, automated counts were well correlated with tumor outcome, manual counts were not.

A second experiment done in collaboration with Darzynkiewicz examined only cells remaining in suspension after monolayering.

Table 6, with one exception, shows a positive correlation between elevated red fluorescence and tumor progression, especially for animals with maximal tumors when samples were

Table 5. Elevated Red Fluorescence of Acridine Orange Stained Leukocytes in Tumor-Bearing Hosts, I.

Host	Tumor Score At Time of Sampling	Response Of Host To Tumor	% of Cells with Elevated Red Fluorescence (Not Monolayered)	(Monolayered)
1	0[1]	--	2.8	1.3
2	0	--	2.3	1.3
3	0	--	7.3	NT[2]
4	0	--	3.6	NT
5	0	--	1.9	NT
6	4	Regression	1.1	NT
7	3	Regression	1.7	1.2
8	6	Died	16.1	11.8
9	6	Died	13.0	8.4
10	6	Regression	1.8	1.0

[1]0, Uninoculated Controls

[2]NT, Not Tested

Table 6. Elevated Red Fluorescence of Acridine Orange Stained Leukocytes in Tumor-Bearing Hosts, II.

Host	Tumor Score At Time Of Sampling	Response of Host to Tumor	% Cells With Elevated Red Fluorescence
11	0[1]	--	0.8
12	0	--	6.9
13	0	--	3.4
14	2	Died	8.6
15	2	Regression	2.3
16	6	Died	17.1
17	6	Died	10.2
18	6	Died	18.3
19	6	Regression	24.4
20	6	Died	44.4

[1]0, Uninoculated Controls

taken. Host number 19, whose wing tumor regressed, died shortly after the experiment ended. Since no necropsy was performed, the presence or absence of visceral metastasis is unknown, and its elevated count remains an enigma.

It is, therefore, evident that leukocytes from the peripheral blood of chickens with large RSV-induced tumors had elevated red fluorescence relative to controls. It is assumed that this increase in red fluorescence is due to an increase in lymphocyte RNA, and is a result of tumor- and/or helper-virus pathogenesis. This point will be discussed more fully in later sections.

CORRELATION OF RED AND GREEN FLUORESCENCE WITH DIFFERENTIAL CELL-TYPE.

Additional automated cytophotometric analyses were done by Dr. J. Leary, Los Alamos Scientific Laboratory, Los Alamos, N.M. These samples, which were fixed in glutaraldehyde as described earlier, confirmed elevated red fluorescence in cells from animals with large progressive tumors. Further, comparison of differential counts with histograms generated by the cell cytophotometer allowed assignment of given cell types to particular peaks of red fluorescence. Histograms from samples with greater than 80% red blood cells are shown in Fig. 5, indicating that erythrocytes have the lowest red fluorescence of blood cells. Histograms in Fig. 6 represent samples with a low lymphocyte to thrombocyte ratio, and in Fig. 7 samples with a high lymphocyte to thrombocyte ratio. These figures indicate that lymphocytes had more intense red

Fig. 5. Histograms generated from automated analysis of red and green fluorescence after acridine orange staining of cells. I. Both samples contain greater than 80% erythrocytes.

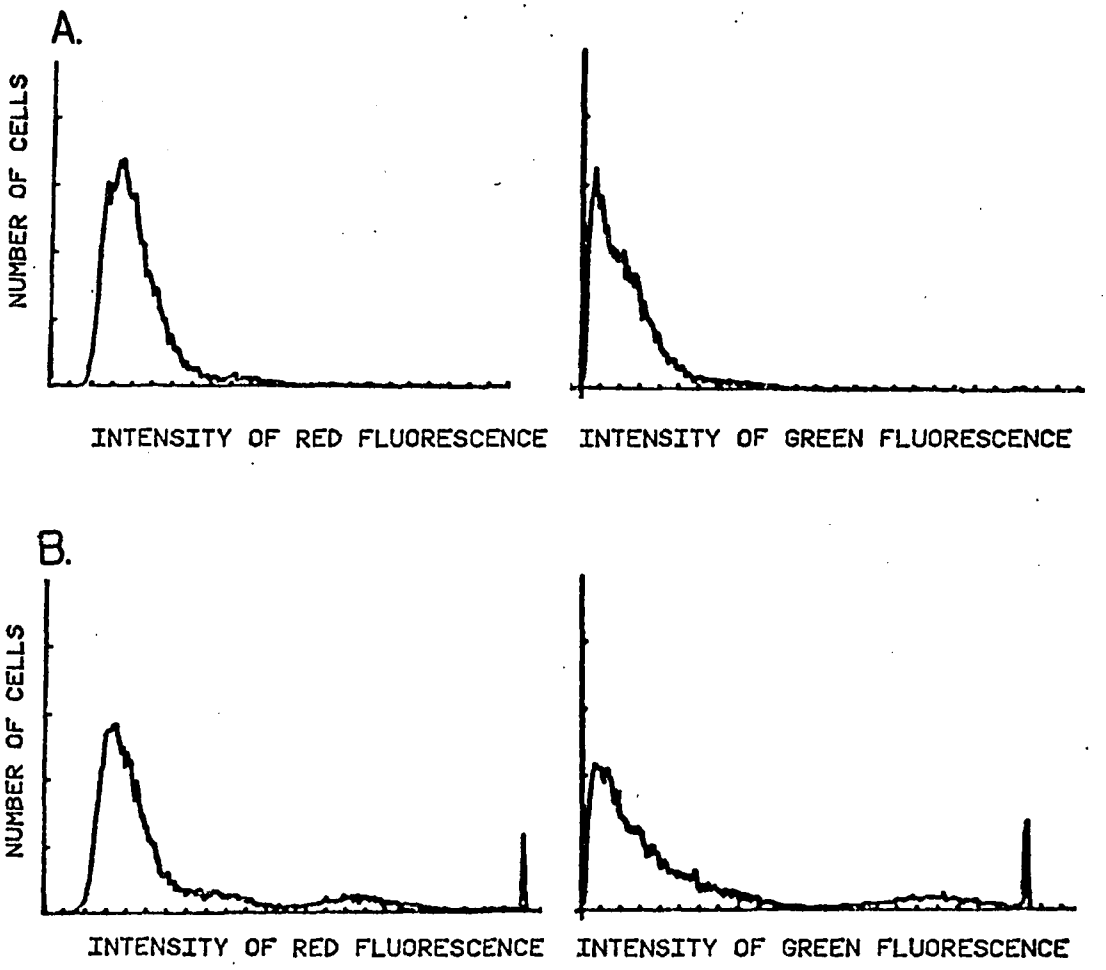

A.
NUMBER OF CELLS
INTENSITY OF RED FLUORESCENCE
INTENSITY OF GREEN FLUORESCENCE
B.
NUMBER OF CELLS
INTENSITY OF RED FLUORESCENCE
INTENSITY OF GREEN FLUORESCENCE

Fig. 6. Histograms generated from automated analysis of red and green fluorescence after acridine orange staining of cells. II. Sample A, 20% lymphocytes, 70% thrombocytes; Sample B, 25% lymphocytes, 50% thrombocytes.

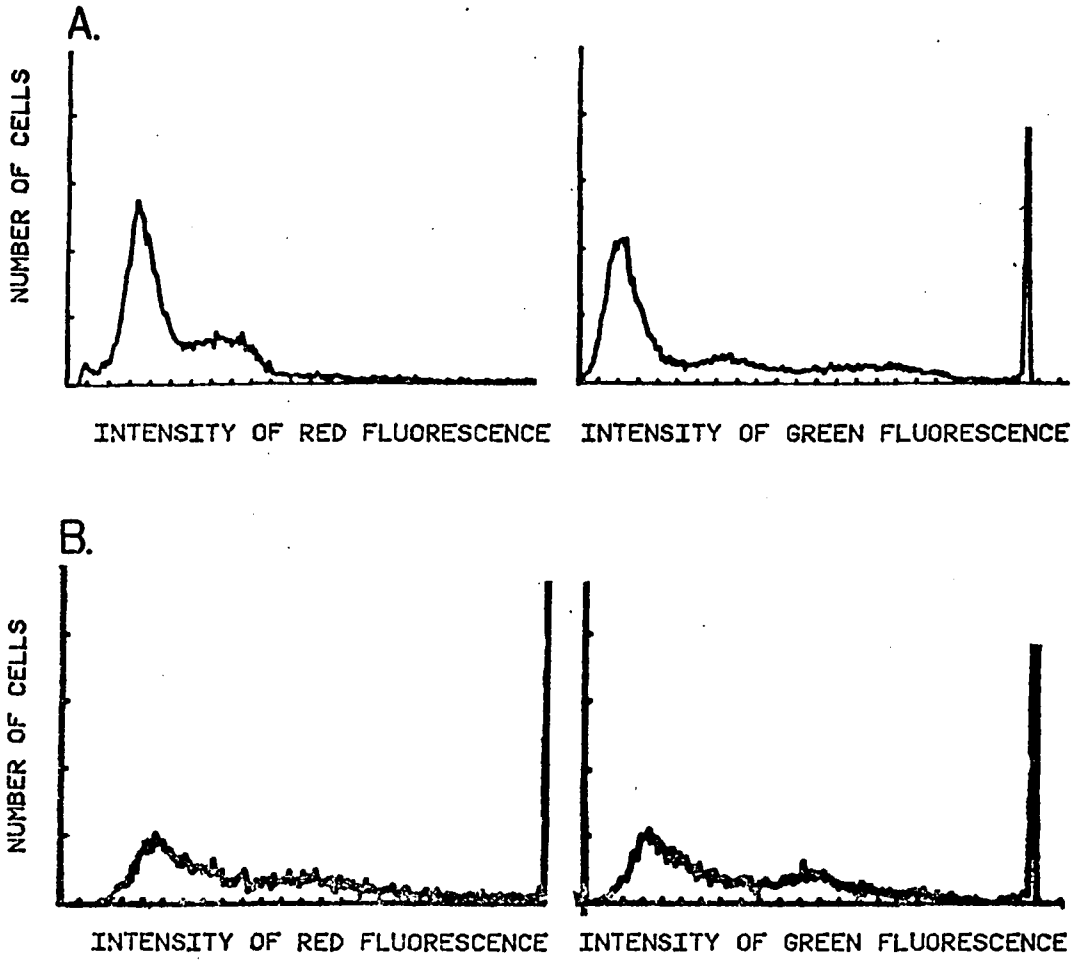
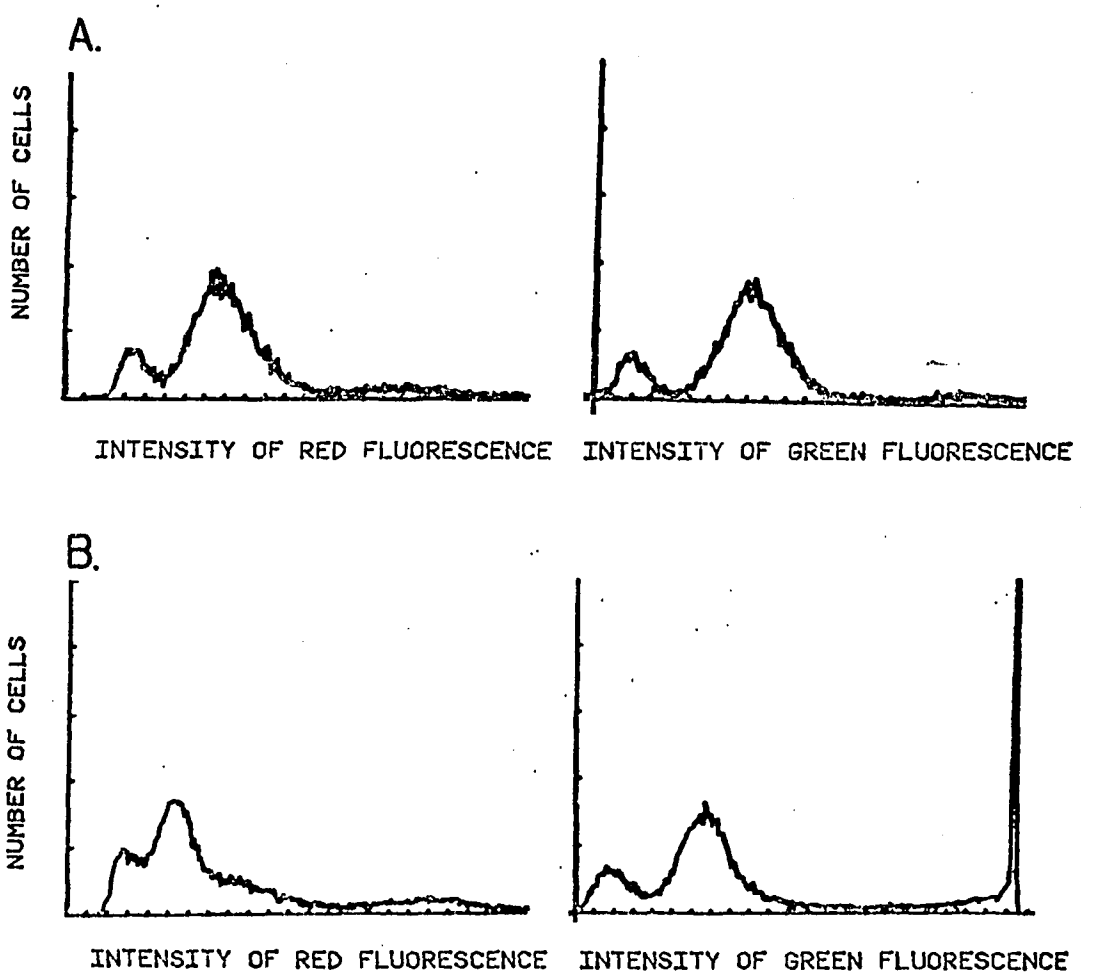

A.
NUMBER OF CELLS
INTENSITY OF RED FLUORESCENCE
INTENSITY OF GREEN FLUORESCENCE
B.
NUMBER OF CELLS
INTENSITY OF RED FLUORESCENCE
INTENSITY OF GREEN FLUORESCENCE

Fig. 7. Histograms generated from automated analysis of red and green fluorescence after acridine orange staining of cells. Sample A, 85% lymphocytes, 10% thrombocytes; Sample B, 60% lymphocytes, 25% thrombocytes.


A.
NUMBER OF CELLS
INTENSITY OF RED FLUORESCENCE
INTENSITY OF GREEN FLUORESCENCE
B.
NUMBER OF CELLS
INTENSITY OF RED FLUORESCENCE
INTENSITY OF GREEN FLUORESCENCE

fluorescence than erythrocytes, with thrombocytes intermediate between the two.

The above results suggest that neither erythrocytes nor thrombocytes could account for the elevated red fluorescence of cells from tumor-bearing hosts. Since samples were not significantly contaminated by cells other than these, cells with elevated fluorescence were apparently lymphocytes and/or monocytes. Such cells which remained after monolayering were evidently lymphocytes.

RELEASE OF NUCLEIC ACID FROM LEUKOCYTES BY WASHING

Cucchiara (1976) reported that washing lymphocytes with PBS released RNA from spleen cells of birds with large tumors, but not from cells of non-tumorous hosts. He correlated this release with loss of red fluorescence and concluded that RNA was released from the exterior of the cytoplasmic membrane. A washing experiment was done according to Cucchiara (1976) in an attempt to isolate and characterize this RNA. Table 7 indicates that material which absorbed light at both 260 and 280 nm appeared in the supernatant fluid from cell washes, whether cells were from tumorous or non-tumorous hosts. To determine whether this material could have been liberated by cell death and/or lysis, another experiment was done in which cell numbers and viability were monitored. Table 8 shows that greater than 36% of cells were lost, presumably through lysis, after 3 washings.

Table 7. Light Absorbance at 260 nm (A_{260}) or 280 nm (A_{280}) of Supernatant PBS after Washing of Leukocytes and Centrifugation at 400xg for 10 min.

Wash #	Splenic Lymphocytes From 2 Tumorous Hosts		Splenic Lymphocytes From 1 Uninoculated Host	
	A_{260}	A_{280}	A_{260}	A_{280}
1	1.87	1.18	1.37	0.88
2	1.10	0.77	0.67	0.48
3	1.08	0.83	0.68	0.56

Table 8. Cell Viability, Total Cell Number, and Light Absorbance at 260 nm (A_{260}) of Supernatant PBS After Washing of Leukocytes and Centrifugation at 400xg for 10 Min.

	Lymphocytes From 3 Tumorous Hosts			Lymphocytes From 3 Uninoculated Hosts		
Wash #	Total[1] Count	Percent[1] Viability	A_{260}[2]	Total Count	Percent Viability	A_{260}
1	5.4×10^6	81.5	0.70	1.7×10^7	86.9	2.85
2	4.2×10^6	76.2	ND[3]	1.4×10^7	83.2	ND
3	2.9×10^6	69.0	0.09	1.1×10^7	84.1	0.12

[1]Total count and viability determined prior to centrifugation

[2]A_{260} determined from cell supernate following centrifugation

[3]ND, Not Determined

While the amount of 260 nm absorbing material was roughly proportional to the number of cells lysed, more material was liberated per cell lost in earlier than in later washes. A reproducible decrease in red fluorescence of AO stained, washed cells was not detected by microscopic observation. Automated cytophotometry was not used to examine washed lymphocytes.

ABSENCE OF DIFFERENT SERUM PROTEINS IN TUMOR-BEARING AND NON TUMOR-BEARING HOSTS

Cucchiara (1976) reported that chickens with maximal tumors had a unique serum protein which was absent in uninoculated controls. In an attempt to isolate and characterize this protein, SDS-PAG electrophoresis of serum samples was done according to Cucchiara. Seventy-five samples from tumorous animals and 102 from controls were examined, usually in duplicate or triplicate. No consistent difference was observed in the serum proteins of tumor-bearing and control chickens.

EFFECT OF GENOTYPE ON THE DEVELOPMENT OF IMMUNITY

SIMILAR STIMULATION BY T-CELL MITOGENS OF LYMPHOCYTES FROM NORMAL B^2B^2 AND B^5B^5 CHICKENS.

The proliferation of thymus-derived (T) lymphocytes following stimulation with the T-cell specific mitogens con A and PHA was examined. Lymphocytes from 3 chickens per haplotype were tested separately in most experiments as described. While

replicates of the same sample were very reproducible, a wide variation occurred among birds of the same genotype. Therefore, while Table 9 shows some large differences in mean stimulatory index (SI) between B^2B^2 and B^5B^5 chickens, only in the con A assay of experiment 3 was the difference statistically significant ($P < .05$).

SIMILAR NUMBERS OF PERIPHERAL LYMPHOCYTES IN NORMAL B^2B^2 AND B^5B^5 CHICKENS.

During preparation of lymphocytes for mitogen studies, the number of cells isolated per ml of blood was recorded. Table 10 shows that in 4 experiments, the mean cell yield from B^2B^2 birds was slightly higher than from B^5B^5 animals. Only in experiment 2 was the difference statistically significant ($P < .05$). Therefore, prior to tumor induction, B^2B^2 and B^5B^5 hosts appeared to have equal numbers and mitogenic reactivity of peripheral lymphocytes.

GENOTYPE ASSOCIATED RESISTANCE OR SENSITIVITY TO SECONDARY TUMOR-INDUCTION.

McBride et al. (1977) reported that chickens with a progressing RSV-induced tumor in one wing were resistant to a subsequent challenge in the second wing between 8 and 18 days after the first inoculation. However, in animals which resisted the second challenge, accelerated growth of the primary tumor was observed. The method of McBride et al. was used to determine whether or not anti-tumor immunity and/or tumor enhancement develop similarly in RPRL chickens. Six to 8-week

Table 9. Mean Stimulatory Indexes (SI) of B^2B^2 and B^5B^5 Lymphocytes After Culture With the T-Cell Mitogens Concanavalin A (Con A) and Phythomegagglutinin (PHA)

	SI con A		SI PHA	
Experiment #	B^2B^2	B^5B^5	B^2B^2	B^5B^5
1	7.02(3)	10.91(3)	2.64(3)	2.03(2)
2	21.05(4)	9.47(4)	6.64(4)	3.08(3)
3	18.5 (4)[2]	73.25(2)[2]	7.77(5)	9.51(2)
4	4.6 (3)	10.63(3)	3.14(3)	3.59(3)

[1]Numbers in () = # samples assayed

[2]$P < .05$ by one-way analysis of variance

Table 10. A Comparison of the Lymphocyte-Yield per ML of Blood from B^2B^2 or B^5B^5 Chickens.

Experiment #	Mean Lymphocyte Yield Per ml of Blood x 10^6	
	B^2B^2	B^5B^5
1	2.37(3)	1.37(3)
2	6.83(3)[1]	3.27(3)[1]
3	3.37(3)	3.25(2)
4	8.23(3)	7.77(3)

[1] $P < .05$ by one way analysis of variance

chickens of both genotypes (B^2B^2 and B^5B^5) were inoculated with about 20 PFU RSV-1, then challenged with the same dose between days 9 and 12 following the first inoculation. B^5B^5 chickens were not immune, since they invariably developed a tumor in both wings; 94.7% of B^2B^2 birds were resistant to the second challenge (Table 11). Without exception, tumors which developed in B^2B^2 animals underwent spontaneous regression. In four experiments, subjective tumor scores indicated no enhancement of primary tumor growth by a second virus challenge. In fact, accelerated regression by the regressor genotype (B^2B^2) was observed (Fig. 8).

Two additional experiments were performed to more objectively monitor tumor growth. Chickens were inoculated in the left wing at 8 weeks of age, and about half were challenged 12 days later in the right wing. A caliper was used to record the length, width and thickness of the primary tumor between 10 and 28 days following the first inoculation. Tumor volume was determined according to the formula for an ellipsoid ($4/3\ \pi abc$) where a, b and c represent radii.

In these experiments, mean primary tumor volumes of animals which received a single inoculation did not differ significantly from those which were rechallenged (Figs. 9 and 10). In both cases, tumors of B^5B^5 chickens increased exponentially until death of the host at 23 to 30 days post-inoculation (PI). Tumors of B^2B^2 chickens began to regress between 14 and 17 days PI. This indicated, in apparent contradiction of McBride *et al.* (1977) that secondary inoculation had no effect on growth of primary tumors. It should be

Table 11. The Proportion of B^2B^2 and B^5B^5 Chickens Which Developed Tumors in Left Wing Inoculated With RSV-1 at Day 0 and Right Wing Inoculated at Day 10 Through 12.

Genotype	Exp. #	Interval Between Inoculation (Days)	Proportion of Birds That Developed Tumors in	
			Left Wing	Right Wing
B^2B^2(1)	1	10	8/8	1/8
	2	10	6/6	1/6
	3	10	5/5	0/5
	4	12	9/9	0/9
	5	12	10/10	0/10
TOTAL			38/38	2/38
B^5B^5(2)	1	10	4/4	4/4
	2	10	4/4	4/4
	3	10	5/5	5/5
	4	12	7/7	7/7
	5	12	5/5	5/5
TOTAL			25/25	25/25

(1) In every case, the tumor(s) regressed completely

(2) In every case, the tumor grew progressively and the animal died before 36 days post inoculation

Fig. 8. Mean primary tumor scores for chickens inoculated in one wing, or two wings at 10-12 day intervals; combined data for four experiments. ⊡ B^2, 1 wing, N = 24; ■ B^2, 2 wings, N = 25; △ B^5, 1 wing, N = 21; ▲ B^5, 2 wings, N = 21.

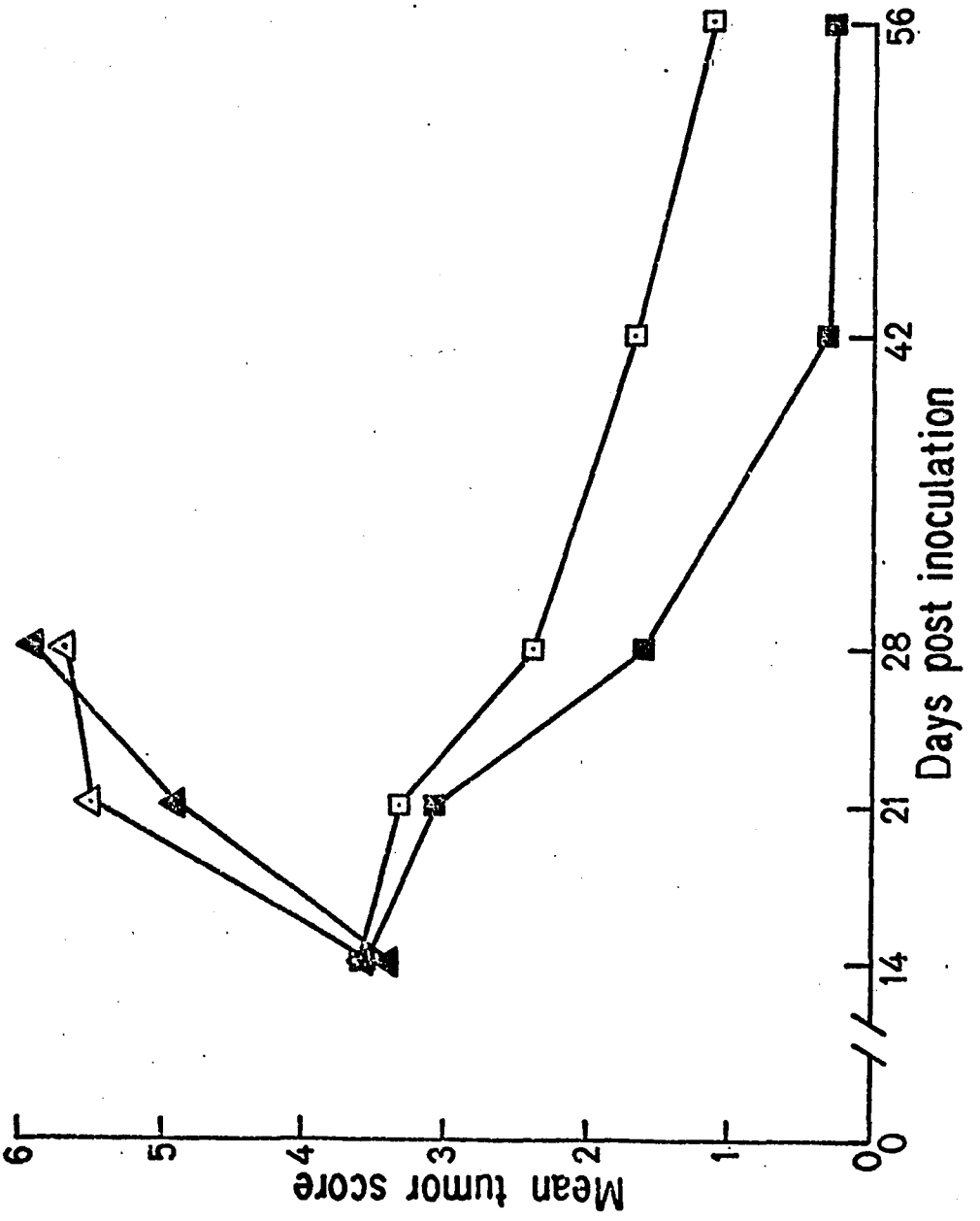

Mean tumor score
0
1
2
3
4
5
6
0
14
21
28
42
56
Days post inoculation

Fig. 9. Mean primary tumor volumes for chickens inoculated in one wing, or both wings at 12 day intervals. I. ⊡, B^2, 1 wing, N = 6; ■, B^2, 2 wings, N = 8; △, B^5, 1 wing, N = 4; ▲, B^5, 2 wings, N = 5.

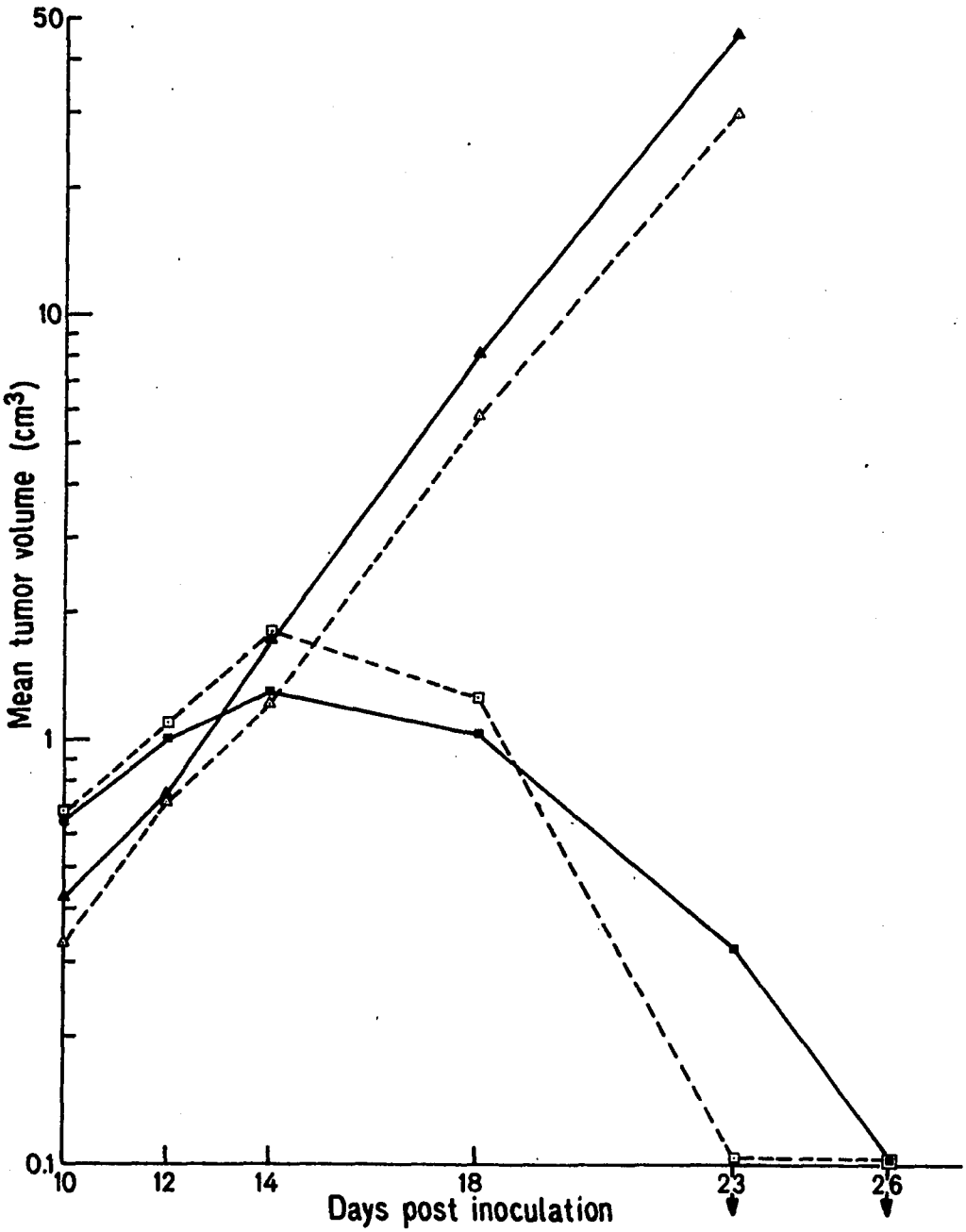
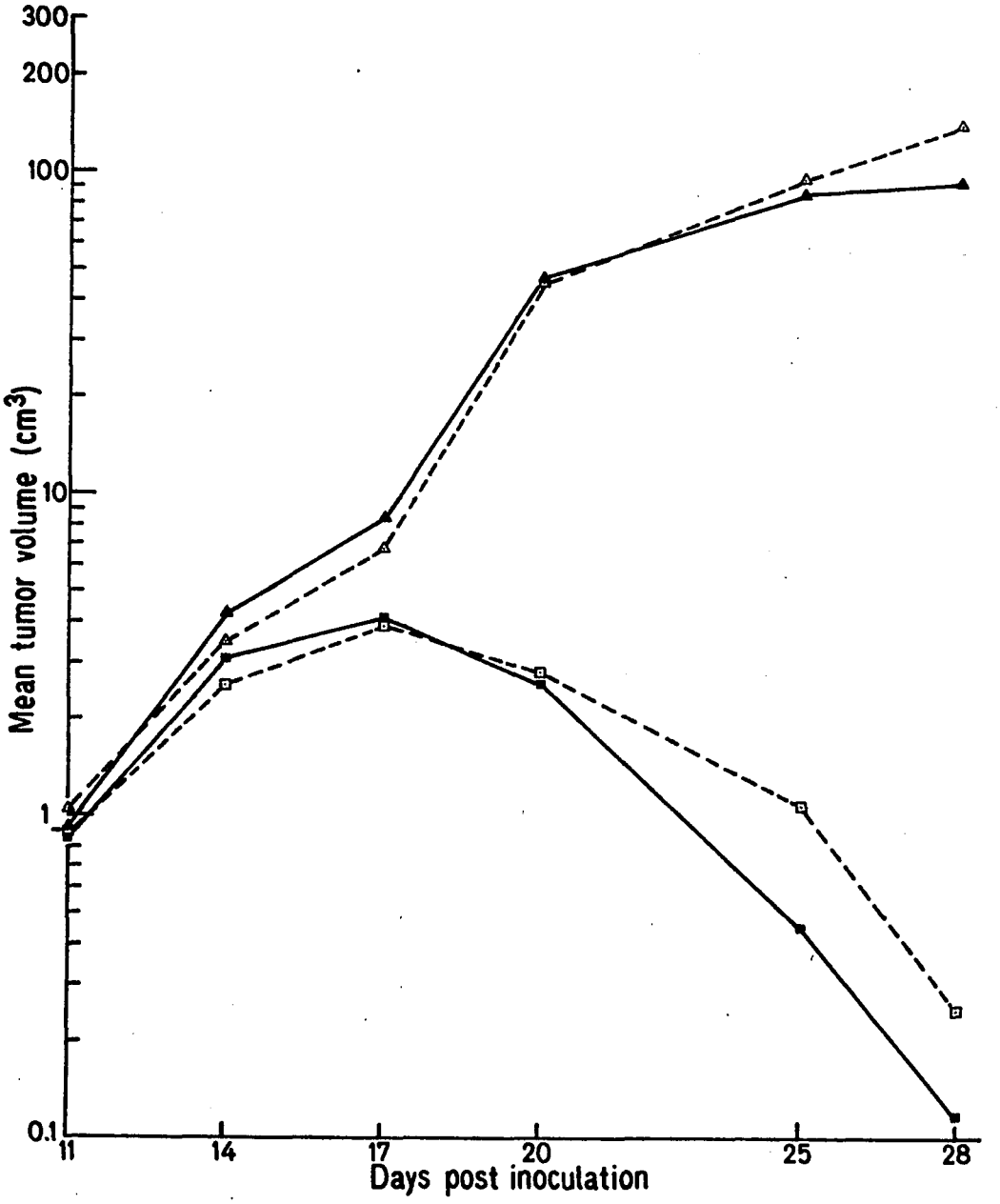

50
10
1
0.1
Mean tumor volume (cm³)
10
12
14
18
23
26
Days post inoculation

Fig. 10. Mean primary tumor volumes for chickens inoculated in one wing, or both wings at 12 day intervals. II.
⊡, B^2, 1 wing, N = 6; ■, B^2, 2 wings, N = 8;
△, B^5, 1 wing, N = 4; ▲, B^5, 2 wings, N = 5.


300
200
100
10
1
0.1
Mean tumor volume (cm3)
11
14
17
20
25
28
Days post inoculation

noted, however, that genetic stocks of both virus and host were different in these two studies.

NEUTRALIZATION OF VIRUS BY REGRESSOR SERUM

Pooled serum from 5 birds which had completely regressed primary tumors was tested for neutralization of RSV-1 according to Whitfill et al. (1978). Blood was collected from the brachial vein, clotted at 37C for 1 h, refrigerated for 1 h, and the serum harvested and pooled. Five-tenths ml of serum was mixed with an equal volume of a $5x10^{-2}$ dilution of RSV-1, and incubated at room temperature for 45 min. Hyaluronidase in the virus diluent was twice the usual concentration prior to mixing with serum. After incubation, 0.1 ml of virus-serum mixture was inoculated into the wing webs of each of 6 susceptible animals, none of which developed tumors. Virus treated identically with serum from "normal" chickens produced tumors in each of 8 birds inoculated. Therefore, serum from regressors neutralized RSV-1 while serum from uninoculated controls did not.

EFFECT OF GENOTYPE ON DEVELOPMENT OF HUMORAL ANTI-VIRAL IMMUNITY.

To determine whether or not humoral neutralization of virus in vivo could be responsible for the failure of secondary tumors to develop at 10-12 days PI, sera from 10 B^2B^2 and B^5B^5 tumor-bearing birds were tested for serum neutralization. Serum from one uninoculated animal per genotype was tested as a control. Table 12 shows that sera from all B^5B^5 chickens

Table 12. Tumor Development in Left Wing Inoculated With RSV on Day 0, and Right Wing Inoculated on Day 12, Plus Detection of Antiviral Antibody on Days 12 and 20.

B-Genotype	Number of Hosts	Fraction With Tumor in Left Wing	Fraction With Tumor in Right Wing	Fraction With Antiviral Antibody at 12 Days PI	Fraction With Antiviral Antibody at 20 Days PI
22	10	10/10	0/10	3/10	NT
55	12	12/12	12/12	0/12	8/8

tested, and from 70% of B^2B^2 birds failed to neutralize the virus. Although over two-thirds of B^2B^2's tested lacked detectable anti-viral antibody, all were resistant to secondary tumor-induction. It, therefore, seems unlikely that *in vivo* neutralization of virus by antibody is responsible for the observed resistance of B^2B^2 chickens at 12 days PI. This concurs with the work of McBride *et al.* (1977), since neonatal bursectomy did not alter development of immunity to a second challenge.

Retarded development of humoral anti-viral immunity occurred in B^5B^5 hosts, with virus-neutralizing activity present at 20 but not 12 days PI. B^5's were not tested for anti-tumor immunity *in vivo* after day 12 PI, since the latency period for tumor-development would then extend into the expected mortality-period (days 22-28 PI).

CHAPTER V

DISCUSSION

The foregoing experiments indicate critically different time courses for the development of anti-tumor and anti-viral immunity in B^2B^2 and B^5B^5 chickens. In the B^2 host, a rapid response to both tumor and viral antigens results in neutralization of virus and regression of tumors. On the other hand, a delayed or suppressed response in the B^5 host results in rapid tumor growth and early mortality.

HOST GENOTYPE, AGE AND TUMOR DEVELOPMENT

A strong association exists in mouse (Shreffler and David, 1975), chicken (Collins et al., 1977) and perhaps human (Dausset, 1977) between major histocompatibility complex (MHC) haplotype and resistance to cancer. The observations of Collins et al. (1977) using F_2 generation chickens, have been extended to F_3 progeny by the present study. B^2B^2 chickens usually developed tumors which regressed, while B^5B^5 animals developed progressive tumors which resulted in early death. Furthermore, neither age at inoculation (from 6 to 8.5 weeks) nor source of the B^2 allele (strain 6_3 or 6_1) had an effect on tumor outcome. Therefore, the gene(s) responsible for the observed phenotypes is probably identical in lines 6_1 and 6_3.

LYMPHOCYTE PURIFICATION AND MITOGEN STUDIES

Lee (1974) noted the importance of removing thrombocytes from lymphocyte preparations which were to be assayed for stimulation by mitogens. She was unable to detect stimulation of cells purified by either albumin flotation or ficoll gradient. In an experiment preliminary to the present study (unpublished), lymphocytes were purified from a split blood sample by both ficoll-diatrizoate (FD) and slow centrifugation. Only lymphocytes from the latter method were stimulated by con A. Numerous differential counts and automated cytophotometric analyses confirmed, contrary to Archambault et al. (1976), extensive thrombocyte contamination of FD-separated leukocytes, which may be responsible for the failure of mitogen stimulation. Thrombocytes were also reported to interefere with the mixed leukocyte reaction (Jones, 1977).

While stimulation of cells separated by slow centrifugation was adequate, it should be noted that this method recovered less than 10% of blood lymphocytes. It is unknown whether or not recovery was random, or selective for lymphocytes of a given size, density or type. However, in agreement with what is found in peripheral blood, these cells were nearly all small lymphocytes by morphological criteria and staining reactions. These experiments indicate that 6-week old B^2B^2 and B^5B^5 chickens have normal numbers of functional peripheral T-cells.

LACK OF ANTI-TUMOR IMMUNITY IN B^5B^5 CHICKENS

Either humoral and/or cell-mediated immunity may account for resistance to RSV-induced tumors.

Animals studied by both McBride et al. (1977) and by Gyles et al. (1977), from strains of chickens prone to progressive tumors, all developed immunity to subsequent virus challenge. In the former study, all birds were immune by 8 days post-inoculation (PI), even after effective neonatal bursectomy. In the latter investigation, 33% were immune at 10 days PI, and 100% at 15 days PI. McBride et al. (1977) observed a bursal dependent enhancement of primary tumor growth following secondary virus challenge.

Neither anti-tumor immunity nor enhancement of tumor growth were observed in B^5B^5 chickens in the present investigation. Furthermore, RSV-neutralizing antibody was not detected in serum from B^5B^5 chickens at 12 days PI. The absence of early anti-tumor and anti-viral immunity in B^5 animals may be due to several factors, including the following:

1) The B^5B^5 haplotype may be associated with general immuno-incompetence due to retarded development of the immune system. This seems unlikely since the present study shows the number of circulating lymphocytes and their response to mitogens to be similar in B^2B^2 and B^5B^5 chickens at six weeks of age. Also size and gross appearance of thymi and bursae from more than 50 birds of each blood type were similar when removed at three weeks of age for antigen preparations (unpublished). Furthermore, B^5 and B^2 birds rejected allogeneic skin

grafts equally well; complement levels of B^5's were equal to or greater than B^2's at six weeks of age; and total serum IgG was normal for six-week old chickens of both lines 6_1 and 15_1 (Heinzelmann; Miller; Collins and Savage, respectively, personal communications).

2) RSV-determined, tumor-associated antigen(s) which is(are) responsible for prompt rejection by B^2B^2 chickens may be structurally similar to a B_5 antigen. This would account for both prompt rejection of tumors by B^2B^2 animals and lack of reactivity by B^5B^5's. That tumor cell transplants from B^5B^5 birds failed to grow in B^2B^2 hosts, but grew progressively in B^5B^5 birds (unpublished) is consistent with, but hardly supportive of this hypothesis. Also, an experiment by Heinzelmann (personal communication) indicated that little influence on tumor regression in B^2 birds resulted from prior neonatal induction of tolerance to B^5 erythrocytes. What the effect would be of tolerance to B_5 leukocyte antigens is presently unknown.

3) The immune system of B^5B^5, but not B^2B^2 animals, may be seriously debilitated by RSV-1 tumorigenesis or infection. Decreased serum complement levels (Miller, personal communication) and failure to mount a rapid immune response against either virus or tumor, indicate a non-specific immune impairment and thus support this view. This immune impairment may be induced by the tumorigenic Rous sarcoma virus, by the leukosis-causing Rous associated virus (RAV-1) or by unknown factors. More experimentation is needed to either support or refute this hypothesis.

4) A B^5-linked gene(s) may be responsible for specific suppression of anti-tumor immunity. This hypothesis is most consistent with information from the mouse. That either adoptive transfer of lymphocytes (Fujimoto, Greene and Sehon, 1976) or passive transfer of a lymphocyte-derived factor (Greene, Fujimoto and Sehon, 1977) from mice with growing tumors, into previously tumor-immunized mice, will result in enhancement of tumor growth, has been demonstrated. This led to the identification of suppressor T-lymphocytes, and factors produced by them, which specifically repress the immune response to a particular antigen. Both suppressor cells (Murphy et al., 1976) and factor (Greene, Pierres, Dorf and Benacerraf, 1977) contained determinants coded for by the I-J subregion of the H-2 (mouse MHC) complex.

Suppressor activity has been detected in chicken thymocytes (Droege, 1975), and this may be the reason for progressive tumors in B^5B^5 hosts. An early immune response (12 days PI) was lacking in all B^5B^5's tested, to both tumor and intact virus. On the other hand, both anti-viral and anti-tumor immunity was detected at 12 days PI in B^2B^2's. This suggests either a general immune paralysis of tumorous B^5B^5 hosts, or a specific repression of the immune system by suppressor cells. Since humoral anti-viral immunity did ultimately develop in B^5B^5's (20 days PI), suppressor activity may have delayed the immune response in this genotype. Unfortunately, three experiments performed according to Greene (personal communication) failed to detect suppression of the

anti-tumor immunity of B^2B^5 chickens into which B^5B^5 lymphocytes had been adoptively transferred.

5) The most likely explanation for the observed results (which may or may not be involved in 3 or 4 above) is that helper virus (RAV-1) infection or transformation occurs only in cells of B^5B^5 hosts, or to a greater extent than in B^2B^2's. These cells, or factors produced by them, then contribute directly to rapid tumor growth, and/or retard the development of immunity in B^5B^5 chickens. Since susceptibility to avian leukosis viruses (including RAV-1) is probably limited to bursal cells (Dunlop, personal communication), this hypothesis is consistent with the observation of McBride et al. (1977) that enhancement of tumor growth required an intact bursa.

Further support for this hypothesis comes from the work of Meyers and Qualtiere (1977) who showed that congenital infection with avian leukosis virus (ALV) resulted in increased size, rate of growth, and incidence of progression of RSV tumors induced at 3 weeks of age. In that study, ALV-infected animals had normal amounts of antibody to both virus and tumor antigens. This is important in light of results reported in this thesis, and the fact that the earliest cytopathic effects of ALV in vitro require 30 days to develop (Purchase et al., 1977). Histopathological changes in vivo have an even longer (100 days) latency period. Since the majority of B^5B^5 birds died by 28 days PI, enhancement of tumor growth (regardless of mechanism) if present, must be an early consequence of leukosis infection (or transformation).

Whether or not such enhancement is specific to RSV-induced tumors in B^5B^5 hosts might easily be determined by studying the effect of RSV-induced tumorigenesis in one wing on the development of an unrelated tumor in the second wing. Clearly, additional investigation will be required to distinguish the effects of RSV from its helper virus, RAV.

ANTI-TUMOR IMMUNITY OF B^2B^2 CHICKENS

The B^2 haplotype of RPRL chicken lines 6_1 and 6_3 is clearly associated with rapid development of immunity to subgroup A RSV-induced tumors. Tumor regression was consistently observed in B^2B^2 chickens by Collins et al. (1977) and in the present study. This study has confirmed that regression is due to anti-tumor immunity, since secondary tumors failed to become established, and many animals had virus-neutralizing antibody. The thirteen B^2B^2 chickens which died with a progressing tumor (7.1% of all B^2B^2's tested) may have been immunologically compromised from endemic diseases perhaps including congenital ALV infection. It is clear that 10 of these 13 cases occurred during an outbreak of aplastic anemia.

FAILURE OF SECONDARY CHALLENGE TO ENHANCE PRIMARY TUMOR GROWTH

The increased growth rate of primary tumors after a second virus-inoculation, as reported by McBride et al. (1977), was not observed in this study. The discrepancy is probably because these investigators compared the size of left wing tumors in birds with either large (both wings inoculated on

day 0) or small (second wing inoculated on day 8) right wing tumors. The tumor load in the former animals was much higher than in the latter, which may have resulted in slower growth of the measured tumor in the former (day 0) animals. The present study indicated no enhanced growth of primary tumors in animals inoculated in the second wing, compared with the normal rate of growth in animals with a single tumor.

ELEVATED RED FLUORESCENCE OF LEUKOCYTES FROM HOSTS WITH PROGRESSIVE TUMORS

Cucchiara (1976) reported an increase in the number of acridine orange stained lymphocytes which fluoresced red concomitant with tumor development. Since all lymphocytes contain RNA, his procedure apparently lacked the sensitivity required to detect low intensity red fluorescence present in all cells. The method of Darzynkiewicz *et al.* was subsequently used, and showed all cells to fluoresce red, some more than others.

While more cells with bright red fluorescence were observed in tumorous than in control animals, manual counting of cells was subjective and very variable. Acridine orange stained cells were therefore analyzed in an automated cytophotometer, which objectively measured fluorescence intensities of individual cells. The results indicated that, indeed, tumorous animals had an increase in the number of peripheral leukocytes with bright red fluorescence. These leukocytes were apparently lymphocytes, since they were mononuclear, nonadherent, and agranulocytic, and were found in the lymphocyte

peak of histograms from automated cytophotometry. That as many as 40% of leukocytes had bright red fluorescence, while more than 80% of cells analyzed were either thrombocytes or lymphocytes, also supports this conclusion.

Cells with bright red fluorescence have an increased RNA content (Darzynkiewicz et al., 1976) associated with RNA and protein synthesis. In tumor birds, this may be indicative of active suppressor cells, virus infected, or transformed cells. Since peripheral leukocyte preparations from animals with large tumors produced sarcomas in susceptible chicks, with a latent period shorter than that for virus (unpublished results), transformed cells were apparently present in peripheral blood. Since these red fluorescing cells were not removed by monolayering, they were apparently not fibroblasts, and probably represented either virus-infected or transformed lymphocytes. They may be involved in non-specific immunosuppression, suppression specifically directed at anti-tumor immunity or in the production of tumor enhancing (growth) factors.

NUCLEIC ACID RELEASED BY WASHING

Cucchiara (1976) associated loss of red fluorescence after washing of lymphocytes with the appearance in washes of RNA. He was able to wash A_{260} absorbing material from lymphocytes of tumor-bearing birds only. However, he did not monitor cell viability or numbers subsequent to repeated washes.

In experiments with both splenic and peripheral lymphocytes, the present results indicate loss of up to 47%

of cells after three washes. A_{260} material was found in washes from both normal and tumor-bearer lymphocytes, in complete disagreement with Cucchiara, and probably represented intracellular nucleic acid liberated by cell-lysis.

SUMMARY

Cells (probably lymphocytes) with elevated RNA levels were detected in the peripheral blood of chickens with large RSV-induced tumors and probably represented infected or transformed cells. This agreed with the finding that chickens susceptible to progressive tumors (B^5B^5 genotype) appeared to be immunologically impaired following, but not prior to, tumor induction. Chickens of the regressor genotype (B^2B^2) had functional immunity at 10-12 days PI since they resisted a second virus challenge and usually had virus-neutralizing activity in their sera. That not all tumor-resistant animals had detectable anti-viral antibody suggested that immunity to RSV-induced tumors was not antibody dependent.

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APPENDIX

I. Diluent for Virus

A. Formula

Hanks balanced salt solution (GIBCO).....92 ml

Fetal calf serum (GIBCO)..... 5 ml

Penicillin (GIBCO), 10,000 u/ml..... 1 ml

Streptomycin (GIBCO), 10,000 ug/ml..... 1 ml

Hyaluronidase, 1000 ug/ml..... 1 ml

B. Preparation

The above sterile ingredients were mixed aseptically and the diluent stored in 50 ml aliquots at -20 C.

II. Ficoll Diatrizoate

A. Formula

24 parts of 9% (w/v) Ficoll (m.w. 40,000; Sigma)

15 parts of 33.9% (w/v) hypaque brand sodium diatrizoate (Winthrop)

B. Preparation

Ficoll and hypaque stocks were prepared separately, mixed, distributed into glass bottles, and autoclaved at 110 C for 30 min. Specific gravity was 1.085-1.090. Unopened bottles were stored at room temperature.

III. Glutaraldehyde Fixative for Acridine Orange Staining

A. Formula

NaH_2PO_4 (2.26% w/v; Baker)	96.2 ml
NaOH (2.52% w/v; Baker)	3.8 ml
Glutaraldehyde (25% w/v; EMS)	18.0 ml

B. Preparation

Stock solutions of sodium phosphate and sodium hydroxide were stored at 4 C for up to 3 months. Salts were mixed, glutaraldehyde was added, and fixative was stored tightly sealed for up to 1 month.

IV. Natt-Herrick Stain

A. Formula

NaCl (Baker)	3.88 g
Na_2SO_4 (Baker)	2.50 g
Na_2HPO_4 (Baker)	1.15 g
KH_2PO_4 (Baker)	0.25 g
Formalin (37%)	7.50 ml
Methyl violet 2B (Baker)	0.10 g

B. Preparation

The above chemicals were dissolved in distilled water in the order listed and diluted to a total volume of 1,000 ml. After standing overnight, the solution was filtered through Whatman no. 2 paper, and was ready for use. The stain was stored at room temperature in the dark for up to 1 year.

C. Use

Cells were diluted 1:10 or 1:100 into stain, shaken for 1 min, and observed in a wet mount preparation.